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SOME ASPECTS OF THE GROWTH OF SINGLE AND MIXED POPULATIONS OF FLAGELLATES AND CILIATES

THE EFFECT OF TEMPERATURE ON THE GROWTH OF *CHILOMONAS PARAMECIUM**

BY SMILJA MUČIBABIĆ

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(*Received* 12 *January* 1956)

(With Plate 8)

The effect of temperature has been studied more than that of any other single ecological factor, and its significance for the growth of populations of many species has been known for a considerable period. Previous studies on Protozoa have been confined to the effect of temperature on the rate of division, and so far as is known other aspects of the problem have not as yet been examined. Thus, Woodruff & Baitsell (1911) and Mitchell (1929) examined the effect of temperature on the division rate of *Paramecium* in cultures containing bacteria. This effect was also studied on *Chilomonas paramecium* in isolation cultures by Smith (1940), and on *Tetrahymena geleii* by Phelps (1946). Phelps observed division rates only in the logarithmic phase of growth, and in that work he was not concerned with the effect of temperature on population growth. It seemed worthwhile, therefore, to examine the effect of temperature on the growth of *Chilomonas paramecium* in greater detail.

MATERIAL AND METHODS

Material. The organism used in these experiments was the cryptomonad flagellate *Chilomonas paramecium* Ehrenberg. The strain was obtained from the Culture Collection of Algae and Protozoa, Botany School, Cambridge. Before restarting experiments, a subclone was developed from the culture in the Culture Collection which originated from one individual. This strain was isolated by Pringsheim. It first appears in the list of his culture collection in 1936 and was not mentioned in the list of 1929. Over such a long period variations may well appear in the population. Kidder & Dewey (1945) mention a biochemical variation that arose between the parent strain T of *Tetrahymena pyriformis* and a daughter strain T-P. A fresh clone was therefore established in order to ensure that the populations of *Chilomonas* used in these experiments were homogeneous.

Medium. Stock solutions of 10% beef-extract (Difco) and of 10% sodium acetate were prepared. A few drops of preservative (1 part flortoluene, 2 parts

* Part of a dissertation presented for the degree of Doctor of Philosophy in the University of Cambridge.

n-butyl chloride, 1 part ethylene dichoride) were added to each stock solution. These were stored in the refrigerator at 5–7° C.

Chilomonas was maintained in a medium of 0·1 % beef-extract and 0·1 % sodium acetate. The medium was always made up from these stock solutions and glass-distilled water. To prevent any production and accumulation of bacterial products, the distilled water was sterilized as soon as it had been distilled, and this sterile glass-distilled water was then used for all media. These were autoclaved at 120° C. As soon as the pressure reached 1 atmosphere, the gas was turned off. From start to finish this operation lasted about 30 min.

It is important to mention that the medium was always prepared one day before inoculation, because growth is then better than in a medium that has been kept for some time.

Glassware. All test-tubes, pipettes and watch-glasses were washed with detergent Comprox 'A', rinsed well with tap water and distilled water. They were sterilized in the oven at 120° C. for 2 hr. The preparation of pipettes and watch-glasses for sterilization was carried out as described by Pringsheim (1946).

The volume of the Pyrex-glass test-tubes used was 3 c.c. Their diameter was measured before starting experiments, and only test-tubes 7·5 mm. wide were selected.

The preparation of cultures. Half a millilitre of sterile medium was pipetted into each sterile test-tube. It would have been easier to postpone sterilization until after the delivery of the medium, but this technique was not used so as to avoid the small loss of water consequent on sterilization and the small differences in composition of the medium of individual cultures, which might have appeared had they been sterilized separately.

The inoculum was taken from a culture $2\frac{1}{2}$–3 days old. The inoculum was put into a sterile watch-glass, and under a low power of a dissecting binocular microscope ten organisms were picked up with a capillary micro-pipette and placed in each test-tube. Four to six cultures were usually inoculated from the same watch-glass. Grease-proof paper secured with an elastic band was wrapped over the non-absorbent sterile cotton-wool plug of the test-tube.

The cultures were kept in the dark at eight different temperatures from 5 to 36° C., in air-tight Kilner jars, under water, in tanks maintained at thermostatically controlled temperatures, except for cultures at 10·5° C., which were kept in a constant temperature room. They were inoculated from stock cultures kept at 22·5° C. It was not observed that sudden transfer from 22·5° C. to other temperatures induced a lag phase. Acclimatization has been practised by some authors and may perhaps be important in short experiments in which observations do not last longer than 24 hr., but it is unnecessary in experiments on population growth. The cultures were run for at least several days, one series for over a month and another for about 2 months.

Size measurements. Preliminary measurements of size on fixed organisms were not satisfactory, because the organisms shrank during fixation. Subsequently, photographs of living organisms were taken every second day throughout the period of growth of the population.

In order to reduce the time of exposure as much as possible, so that instantaneous pictures of swimming organisms ($\frac{1}{200}$th sec. exposure) could be obtained, a 250 W. mercury arc lamp was used as the light source. A heat filter (Chance Brothers heat resisting glass, ON 20), an ordinary blue glass and a blue filter (Kodak, Wratten M filters, 47) were placed between the lamp and the microscope, to avoid damage to the organisms during the exposure. Initially the organisms were photographed with a × 10 objective and a × 10 eyepiece, and a Leica camera attachment; but later a low-power objective (× 6) and a high-power eyepiece (× 25) were used, in order to increase depth of focus and so to obtain a higher proportion of sharp photographs of organisms in a single field.

A photograph of a micrometer slide scale was included in each film. The final enlargement of the print was about 300 times.

The size of the organisms—length and greatest width—was measured from photographs with dividers. Only organisms in sharp focus were measured.

Counting. The organisms were counted by pipetting off drops of the culture, taking into account only living individuals. Whole populations were counted as long as the numbers were under 1000; but when greater than this, five samples of standard volume were taken from each culture and counted. In order to check the sampling method the whole population was counted in ten instances, even though the cultures exceeded 1000–8000 organisms. If the sample included more than fifty organisms, the cultures were diluted with the same medium 3, 5, 10, ..., 40 times, and then sampled. Calibrated pipettes with a constriction were used for sampling; at first a pipette of 4·56 mm.^{3} was used, then one of 3·72 mm.^{3} and lastly one of 5·48 mm.^{3}. After the counting the culture was discarded.

I am indebted to Mr P. A. Plack, of the Department of Biochemistry, Cambridge University, for the gift of these pipettes which were made and calibrated by him.

Sterility test. Before starting to count a loop of every culture was spread over a slope of bacto-nutrient agar (Difco). These agar slopes were incubated at 22·5° C. for 2 weeks. Data from contaminated cultures were discarded.

GROWTH OF POPULATION IN TERMS OF THE TOTAL NUMBER OF ORGANISMS

Growth of population did not occur at temperatures of 5 and 36° C., and at 36° C. the organisms died during the first day after inoculation. At 5° C., however, some of them survived, but they did not multiply. For this reason, data for cultures at 5 and 36° C. do not appear in Table 1; figures are given only for the temperatures at which growth occurred. The table shows the average size of population ($\bar{x}$), standard deviation (s)* and number of population examined (n), for each temperature and age. These data are represented graphically in Text-fig. 1, where the logarithm of the total number of organisms is plotted as ordinate and the age of the populations as abscissa. It is clear from the curves that temperature has a profound

* Standard deviation is calculated from the formula $\sqrt{\frac{\Sigma d^2}{n-1}}$.

Table 1. *The growth of populations of* Chilomonas paramecium *at different temperatures in terms of total number of organisms*

Age in days	Temperature																	
	10·5° C.			16° C.			22·5° C.			26° C.			30° C.			33° C		
	$\bar{x}$	s	n	$\bar{x}$	s	n	$\bar{x}$	s	n	$\bar{x}$	s	n	$\bar{x}$	s	n	$\bar{x}$	s	n
1	11·5	2·3	4	23·6	3·2	3	54·8	4·88	103	120	5·5	3	126	15·3	3	33·3	7·6	3
2	15·8	2·4	6	79·25	10·1	4	406	97·2	143	1,490	304	3	1,368	100·4	3	107·6	23·7	3
3	28	4·1	4	171·0	25·6	3	2,724	891	35	10,630	1,890	3	13,400	3,608	3	141	11·1	3
4	37	6·8	5	552·3	70·0	3	14,488	5,090	24	85,000	15,200	5	84,300	5,400	3	86·3	29·0	3
5	46·8	10·8	4	1,440	70	3	59,620	16,500	24	102,200	15,200	5	90,000	3,400	3	26	4·3	3
6	69·5	17·9	4	5,766	934	6	101,000	20,000	10	103,600	16,000	7	98,300	7,300	3	—	—	—
7	87·75	28·6	4	8,600	2,060	4	104,000	10,100	7	118,300	15,100	3	95,600	15,700	3	—	—	—
8	107·5	47·4	4	14,050	3,510	4	103,000	16,800	8	119,000	—	1	95,000	—	1	—	—	—
9	141	20·2	4	28,040	10,970	7	105,500	12,000	4	142,000	—	1	108,000	—	1	—	—	—
10	199·3	22·2	3	53,600	23,800	6	118,300	32,300	3	112,000	4,200	2	—	—	—	—	—	—
11	290	35·0	3	72,600	1,500	3	100,300	15,000	3	78,000	—	1	—	—	—	—	—	—
12	294·2	76·4	5	87,800	6,600	5	152,500	13,300	2	83,000	—	1	—	—	—	—	—	—
13	343	19·5	4	94,000	7,800	3	136,000	2,800	2	—	—	—	—	—	—	—	—	—
14	643	216·2	4	104,500	7,300	4	121,000	—	1	—	—	—	—	—	—	—	—	—
15	830	17·0	3	109,000	—	1	100,000	—	1	—	—	—	—	—	—	—	—	—
16	—	—	—	111,000	—	1	101,000	—	1	—	—	—	—	—	—	—	—	—
17	1,510	606	4	107,000	—	1	66,000	—	1	—	—	—	—	—	—	—	—	—
18	—	—	—	109,000	—	1	36,000	—	1	—	—	—	—	—	—	—	—	—
19	1,898	467	4	103,000	—	1	—	—	—	—	—	—	—	—	—	—	—	—
20	—	—	—	125,000	—	1	—	—	—	—	—	—	—	—	—	—	—	—
21	2,710	753	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22	—	—	—	106,000	—	1	—	—	—	—	—	—	—	—	—	—	—	—
23	5,560	450	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
25	10,060	730	3	145,000	—	1	—	—	—	—	—	—	—	—	—	—	—	—
27	12,700	1,470	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
29	15,700	3,500	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
30	—	—	—	126,000	—	1	—	—	—	—	—	—	—	—	—	—	—	—
31	22,400	7,130	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
33	36,860	3,660	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
35	46,600	16,200	3	113,000	—	1	—	—	—	—	—	—	—	—	—	—	—	—
38	63,000	16,800	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
41	65,600	3,100	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
45	65,600	19,300	3	82,000	—	1	—	—	—	—	—	—	—	—	—	—	—	—
47	72,600	7,500	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
50	68,000	12,200	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

effect on the growth rate of the population, but has no significant effect on their maximum yields. A profound difference in the size of maximum population appeared only at 33° C., where the temperature begins to exert a lethal effect. At this temperature the organisms lived only for 5 or 6 days.

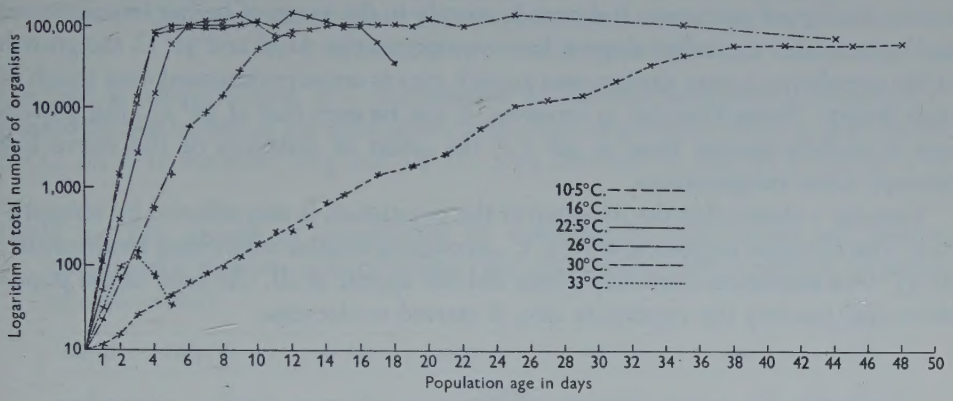

Fig. 1. Effect of temperature on population growth of *Chilomonas* in terms of total number of organisms.

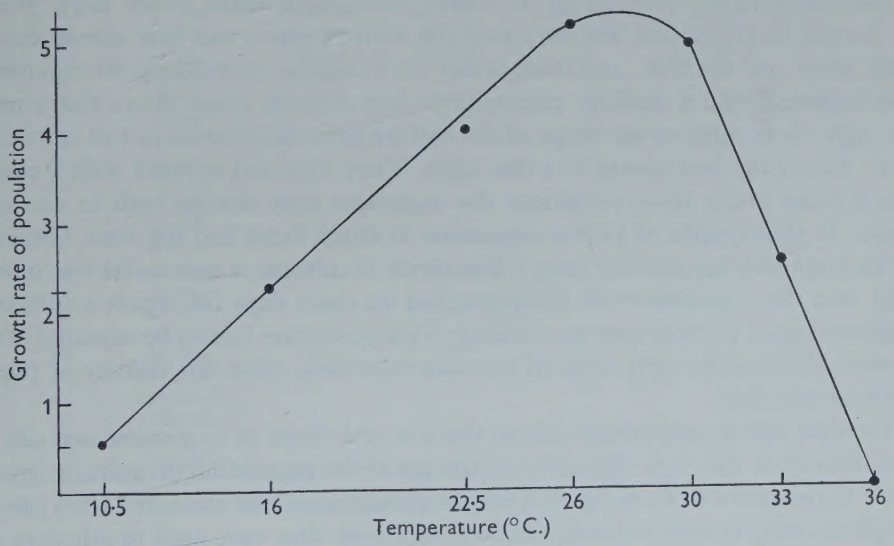

Fig. 2. Relation between temperature and rate of population growth of *Chilomonas*.

Since from the graph in Text-fig. 1 it is not possible to see by inspection what is the relationship between the temperature and the growth rate of population, it was necessary to calculate the growth rates in the logarithmic phase for each temperature, and to plot these values against the temperature. This has been done in Text-fig. 2.

The growth rates are the slopes (that is, the tangents of the angle between abscissa and the growth curve) of the straight lines that represent the growth of population in the logarithmic phase. The rates are plotted on the ordinate and the temperatures on the abscissa. The asymmetrical curve obtained in this way recalls similar curves representing relationships between the temperature and the rate of various biological processes: it descends steeply in the range of higher temperatures and has a smaller ascending slope at lower temperatures. At 26 and 30° C. the growth of the population is very similar, and growth curves are superimposed over much of their length. From Text-fig. 2, however, it can be seen that at 26° C., the growth rate is slightly greater than at 30° C.; the point of inflexion of the curve lies between these temperatures.

Text-fig. 1 shows that the life-span of the population is also affected by temperature. The life-span is greatest at 10·5° C., becoming smaller with rising temperature. At 33° C. a maximum stationary phase did not appear at all. As soon as the population had reached the maximum size, it started to decrease.

GROWTH OF POPULATION IN TERMS OF THE TOTAL VOLUME OF ORGANISMS

Photographs of organisms were taken every second day during population growth at each temperature (only at 33° C. were photographs taken every day). When the density of population was very low, the entire culture was first spread out in small drops over the slide, and then, under the binocular microscope, all organisms were collected with a capillary micropipette into a single drop. When the density was high, about eight to ten drops of the culture were distributed in two rows on a slide. Each drop was spread to a thin layer. They were not covered with a coverslip, because under these conditions the organisms may change both in size and shape. If photographs of twenty organisms in sharp focus had not been obtained for each age and temperature from a first series of cultures, a new series was inoculated, and the organisms were photographed on those days for which a sufficient number of good photographs was lacking. This procedure had to be repeated three or more times in the early stage of population growth, when the density of population is very low.

To what extent temperature affects the size and shape of *C. paramecium* can be seen from Pl. 8, figs. 1–7. The effect of the age of the population (in cultures grown at 30° C.) is shown in Pl. 8, fig. 6. The size of organisms was measured from photographs at different ages and temperatures, and these data were used to calculate the size of the population in terms of total volume (see p. 636). The results are given in Table 2.

The effects of temperature on the maximum size of population expressed as numbers of individuals and expressed as total volume of individuals are not the same, as can be seen from Table 3 and Text-fig. 3; for while the maximum size of populations, expressed as total volume, is decreasing with increasing temperature, the maximum size expressed as total number is increasing. It is to be noticed that

at 10·5° C., a population which is significantly smaller than others in terms of number of organisms, is not merely as large, but in fact larger than the others in terms of total volume.

THE TEMPERATURE COEFFICIENTS AND THE THERMAL INCREMENTS OF THE MULTIPLICATION RATE OF *CHILOMONAS PARAMECIUM*

The rate of multiplication of *C. paramecium* at different temperatures may be expressed by the length of the generation time. This is given in Table 4. The values of Q_{10} are 8·71 between 10·5 and 16° C., 3·55 between 16 and 22·5° C. and 2·03

Table 2. *The growth of populations of* Chilomonas paramecium *at different temperatures in terms of total volume* (*the size of population is given in thousands of cubic micra*)

Days	10·5° C.	16° C.	22·5° C.	26° C.	30° C.	33° C.
0	11	11	11	11	11	11
1	—	—	—	—	—	79
2	34	173	665	2,676	2,179	216
3	—	—	—	—	—	362
4	82	841	15,147	78,391	82,867	225
5	—	—	—	—	—	85
6	164	11,880	97,137	82,685	91,616	—
8	219	21,960	89,481	92,567	—	—
10	454	73,459	82,810	—	—	—
12	743	107,500*	—	—	—	—
14	1,290	111,541	—	—	—	—
16	1,962	—	—	—	—	—
18	2,167	—	—	—	—	—
20	4,427	—	—	—	—	—
22	5,317	—	—	—	—	—
24	11,985	—	—	—	—	—
26	20,226	—	—	—	—	—
28	28,857	—	—	—	—	—
30	47,534	—	—	—	—	—
32	55,175	—	—	—	—	—
34	72,263	—	—	—	—	—
36	93,298	—	—	—	—	—
38	106,895	—	—	—	—	—
40	127,696*	—	—	—	—	—
42	103,673*	—	—	—	—	—
45	116,046	—	—	—	—	—
47	110,352	—	—	—	—	—
50	81,150	—	—	—	—	—

* Interpolated values.

between 22·5 and 26° C. The corresponding values of μ (Text-fig. 4) are 32,891, 21,638 and 12,366 cal. These were calculated according to the formulae:

$$Q_{10} = \frac{10\,(\log k_1 - \log k_2)}{t_1 - t_2}$$

and

$$\mu = \frac{4{\cdot}6\,(\log k_2 - \log k_1)}{1/T_1 - 1/T_2},$$

(Bělehrádek, 1935), where k_1 and k_2 are rates of population growth, t_1 and t_2 centigrade temperatures, and T_1 and T_2 absolute temperatures. Since the values for the generation time are the reciprocals of the number of generations per unit time, the signs in the numerator had to be changed in these calculations.

Table 3. *The effect of temperature on the maximum population of* Chilomonas paramecium

Temperature		10·5° C.	16° C.	22·5° C.	26° C.	30° C.	33° C.
Size of population	Total number of organisms	68,000	114,000	118,000	119,000	98,000	141
	Standard error	900	1,150	2,300	2,800	1,260	3·7
	Total volume of organisms (millions of cubic micra)	113	112	97	93	92	0·36
	Standard error	2·1	—	—	—	—	—

Table 4. *The effect of temperature on the generation time of* Chilomonas paramecium

Temperature (° C.)	Generation time (hr.)
10·5	58·46
16	19·40
22·5	8·517
26	6·649
30	6·931
33	14·0

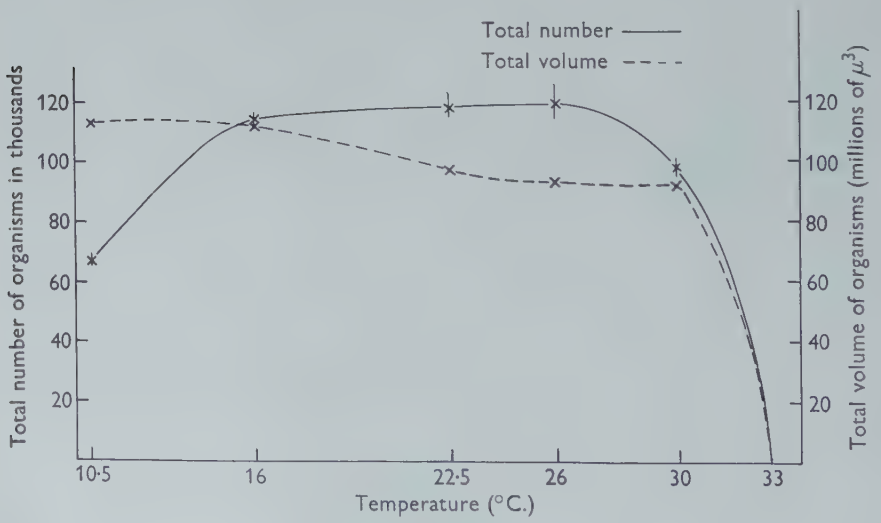

Fig. 3. Relation between temperature and maximum size of population of *Chilomonas* (in terms of total number and total volume of organisms).

In Text-fig. 5 the values of the 'biological zero' (α) was taken into account, according to the formula

$$b = \frac{\log y_1 - \log y_2}{\log (t_2 - \alpha) - \log (t_1 - \alpha)},$$

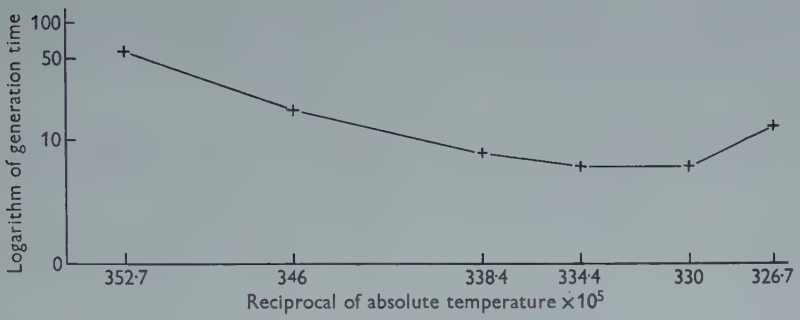

Fig. 4. Logarithm of generation time of *Chilomonas* plotted against reciprocal of absolute temperature × 10^5.

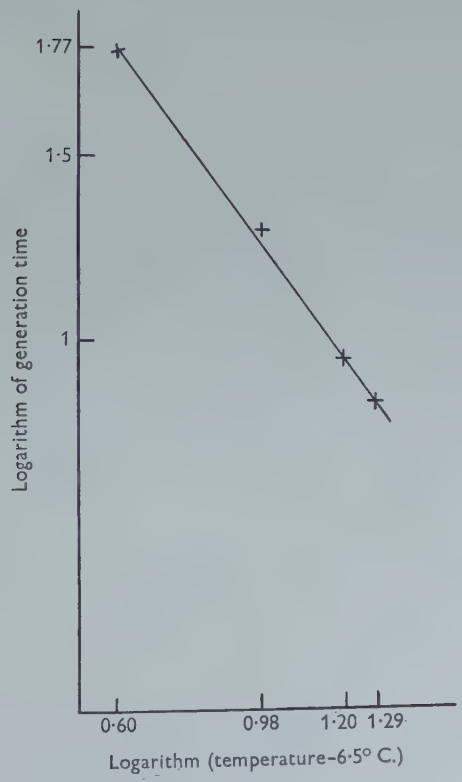

Fig. 5. Logarithm of generation time plotted against logarithm of temperature minus biological zero.

suggested by Bělehrádek (1935). In the present experiments the value of the biological zero was not known, except that it must lie between 10·5 and 5° C., i.e. the lowest temperature which supported the growth of population and the temperature at which only a few organisms survived. Introducing temperatures 5, 5·5, 6, . . . , 9.5 as values for α in this formula, it was found that the value 6·5 gave the best fit. The values for the temperature constant b are then: 1·27 between 10·5 and 16° C.; 1·09 between 16 and 22·5° C.; and 1·25 between 22·5 and 26° C. The mean value is 1·20 with a standard deviation of 0·098.

CHANGES IN SIZE AND SHAPE OF *CHILOMONAS PARAMECIUM* AT DIFFERENT AGES OF THE POPULATION AND AT DIFFERENT TEMPERATURES

During these experiments it was noticed that changes occurred not only in numbers of organisms, but also in their size and shape, and it seemed desirable to give quantitative expression to these. Changes in *size* can be represented by data on the length, width and volume of organisms; and changes in shape can be expressed to some extent by changes in the ratio of length to width. This ratio was calculated for each organism which had been measured. The increase or decrease of this ratio shows whether the organisms are becoming more slender or plumper, and its coefficient of variation may give an idea of the extent to which the shape of the organisms varies at a definite age and temperature.

As the form of *Chilomonas* approximates to an ellipsoid, its volume was calculated according to the formula $V = \frac{4}{3}\pi \frac{1}{2}L(\frac{1}{2}W)^2$, where L and W are the length and greatest width. This is the formula for the volume of an ellipsoid with two equal axes. The breadth and thickness of *Chilomonas* are not, in fact, equal; the former is about four-fifths to five-sixths of the latter. From photographs, however, one can only obtain information about the dimensions along two axes: (*a*) the length; and (*b*) the thickness or breadth, or of something between them (width). Since large numbers of organisms were measured, the chances of measuring breadth or thickness were equal. Using the mean value from these data as a mean transverse dimension does not make a great difference to the results. It can be shown by simple calculation that the difference between the volume of an organism calculated from this formula and from the formula in which three different axes are taken into account will be less than 1%.

Tables 5–8 contain data on mean values and coefficient or variation for length, width and their ratio, and for the average volume. As inoculum was always taken from the stock culture at 22·5° C., photographs of organisms on the day of inoculation were taken only once. This is why the data for day 0 are the same for all temperatures.

The data show a remarkable increase in the size of the organisms during the early growth of the cultures at all temperatures. The coefficient of variation also increases during early growth; for some temperatures it is highest in the early stages of growth. It is interesting to note that a large variation in the size and shape of organisms again appeared when the populations were passing into the maximum

e 5. *Length of* Chilomonas paramecium *at different temperatures and at different ages of population*

ys	10·5° C.			16° C.			22·5° C.			26° C.			30° C.			33° C.		
	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*
0	22·9	9·90	27	22·9	9·90	27	22·9	9·90	27	22·9	9·90	27	22·9	9·90	27	22·9	9·90	27
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	27·1	18·08	39
2	27·5	14·97	22	28·0	10·70	28	25·7	10·63	26	26·0	11·86	27	26·1	9·35	24	26·2	13·74	35
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	29·5	9·49	52
4	28·6	10·10	32	25·7	12·94	20	22·8	11·10	50	21·0	11·38	31	20·8	12·36	17	26·2	16·41	26
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	25·3	19·76	24
6	29·4	10·19	31	28·9	12·27	22	21.0	10·56	53	19·1	10·64	50	21·5	6·56	10	—	—	—
8	26·9	9·23	22	26·6	9·69	27	20·0	10·35	46	19·5	14·09	22	—	—	—	—	—	—
0	29·5	11·02	20	25·7	13·53	68	20·4	12·16	21	—	—	—	—	—	—	—	—	—
2	29·9	11·76	34	25·1	13·80	44	—	—	—	—	—	—	—	—	—	—	—	—
4	29·3	9·85	21	23·2	12·10	21	—	—	—	—	—	—	—	—	—	—	—	—
6	27·7	8·10	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	25·8	10·86	28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	28·3	9·12	36	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	26·6	11·90	116	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	27·8	11·19	50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	28·7	7·05	23	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	30·5	7·91	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	30·0	8·29	28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	26·4	11·96	26	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	28·2	8·68	51	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	26·4	13·76	75	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	26·6	15·68	25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	27·2	18·77	54	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	26·08	15·57	338	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	26·5	12·92	30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	22·6	11·05	53	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

e 6. *Width of* Chilomonas paramecium *at different temperatures and at different ages of population*

ys	10·5° C.			16° C.			22·5° C.			26° C.			30° C.			33° C.		
	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*
0	9·6	6·15	27	9·6	6·15	27	9·6	6·15	27	9·6	6·15	27	9·6	6·15	27	9·6	6·15	27
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	12·9	11·63	39
2	12·2	16·31	22	12·2	13·38	28	11·0	14·40	26	11·5	14·63	27	10·8	9·35	24	12·1	8·26	35
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	12·9	10·08	52
4	12·2	11·43	32	10·7	10·33	20	9·4	2·78	50	9·2	11·24	31	9·5	9·16	17	13·8	13·76	26
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15·7	19·75	24
6	12·4	16·30	31	11·7	11·04	22	9·4	14·22	53	8·9	14·09	50	9·1	6·37	10	—	—	—
8	12·1	10·37	22	10·6	11·90	27	9·1	13·06	46	8·7	14·09	22	—	—	—	—	—	—
0	12·2	13·50	20	10·1	14·27	68	8·1	1·13	21	—	—	—	—	—	—	—	—	—
2	12·7	17·70	34	10·7	13·12	44	—	—	—	—	—	—	—	—	—	—	—	—
4	11·4	13·82	21	9·4	13·77	21	—	—	—	—	—	—	—	—	—	—	—	—
6	11·1	9·46	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	10·7	10·77	28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	11·5	10·49	36	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	9·9	14·36	116	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	10·5	10·02	50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	10·8	2·77	23	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	11·7	10·95	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	13·0	16·05	28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	11·7	8·78	26	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	10·9	9·34	51	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	11·3	14·01	75	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	11·0	18·57	25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	11·8	18·77	54	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	10·8	12·83	38	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	11·3	13·54	30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	10·0	2·99	53	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

stationary phase of growth (40th day at 10·5° C., 12th day at 16° C., 8th day at 26° C.). At the extreme temperatures the value of the coefficient of variation reached its maximum. These were the largest values of the coefficient of variation observed for all cultures at all temperatures.

At the end of growth, the size of the organisms was much smaller than in the early logarithmic phase, except at 33° C. where the width and volume of the organisms were maximal. It is interesting that a very similar effect of temperature on shape and size was noticed in organisms at 5° C. They were also very wide.

Table 7. *Ratio of length to width of* Chilomonas paramecium *at different temperatures and at different ages of population*

Days	10·5° C.			16° C.			22·5° C.			26° C.			30° C.			33° C.	
	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.	*n*	$\bar{x}$	C.V.
0	2·40	11·25	27	2·40	11·25	27	2·40	11·25	27	2·40	11·25	27	2·40	11·25	27	2·40	11·25
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·12	18·40
2	2·28	13·40	22	2·32	12·57	28	2·36	16·10	26	2·29	16·16	27	2·38	11·34	24	2·18	13·30
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·26	11·50
4	2·37	10·55	32	2·42	13·60	20	2·45	11·18	50	2.30	10·87	31	2·23	11·21	17	1·94	22·68
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·68	27·98
6	2·41	14·02	31	2·49	11·08	22	2·28	17·98	53	2·15	13·95	50	2·37	7·17	10	—	—
8	2·25	13·35	22	2·54	12·17	27	2·22	12·93	46	2·24	10·27	22	—	—	—	—	—
10	2·45	11·42	20	2·59	14·93	68	2·53	11·78	21	—	—	—	—	—	—	—	—
12	2·38	12·18	34	2·38	17·14	44	—	—	—	—	—	—	—	—	—	—	—
14	2·58	10·08	21	2·50	11·60	21	—	—	—	—	—	—	—	—	—	—	—
16	2·52	11·90	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	2·45	13·08	28	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	2·47	8·91	36	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22	2·73	15·02	116	—	—	—	—	—	—	—	—	—	—	—	—	—	—
24	2·68	12·31	50	—	—	—	—	—	—	—	—	—	—	—	—	—	—
26	2·66	10·15	23	—	—	—	—	—	—	—	—	—	—	—	—	—	—
28	2·67	13·48	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—
30	2·35	14·47	28	—	—	—	—	—	—	—	—	—	—	—	—	—	—
32	2·25	10·22	26	—	—	—	—	—	—	—	—	—	—	—	—	—	—
34	2·60	10·80	51	—	—	—	—	—	—	—	—	—	—	—	—	—	—
36	2·45	14·69	75	—	—	—	—	—	—	—	—	—	—	—	—	—	—
38	2·44	16·80	25	—	—	—	—	—	—	—	—	—	—	—	—	—	—
40	2·34	15·38	54	—	—	—	—	—	—	—	—	—	—	—	—	—	—
42	2·45	17·96	38	—	—	—	—	—	—	—	—	—	—	—	—	—	—
45	2·35	11·91	30	—	—	—	—	—	—	—	—	—	—	—	—	—	—
50	2·26	11·06	53	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Several photographs were taken of one of them, a peculiar monster: 20 μ long, 19 μ wide and 9 μ thick (Pl. 8, fig. 1). It was found in a culture 2 days old. An organism from another culture 9 days old was 31 μ long and 18 μ wide.

While changes in shape of organisms were relatively small at 22·5, 26 and 30° C., they were very pronounced at the extreme temperatures. It is a feature common to all cultures that the organisms become slender at the end of the logarithmic phase of growth and plump in old cultures in the stationary phase. The largest ratio of length to width was attained at 10·5° C. and the smallest at 33° C., i.e. at low temperature the organisms were most slender and at high temperature most plump.

The kind of change that takes place in the size of the organisms at different temperatures can be seen in Tables 5–8 and Text-fig. 6. These show that the

maximum size (length, width and volume) is reached at extreme temperatures (10·5 and 33° C.), but not at the same phase of growth. At 10·5 C. the maximum length, width and volume are in the phase of negative growth acceleration (28th and 30th day), while at 33° C. these maxima are in different phases: the greatest length is in the phase of negative growth acceleration (3rd day), the greatest width and volume in the so-called logarithmic death phase (Buchanan & Fulmer, 1928).

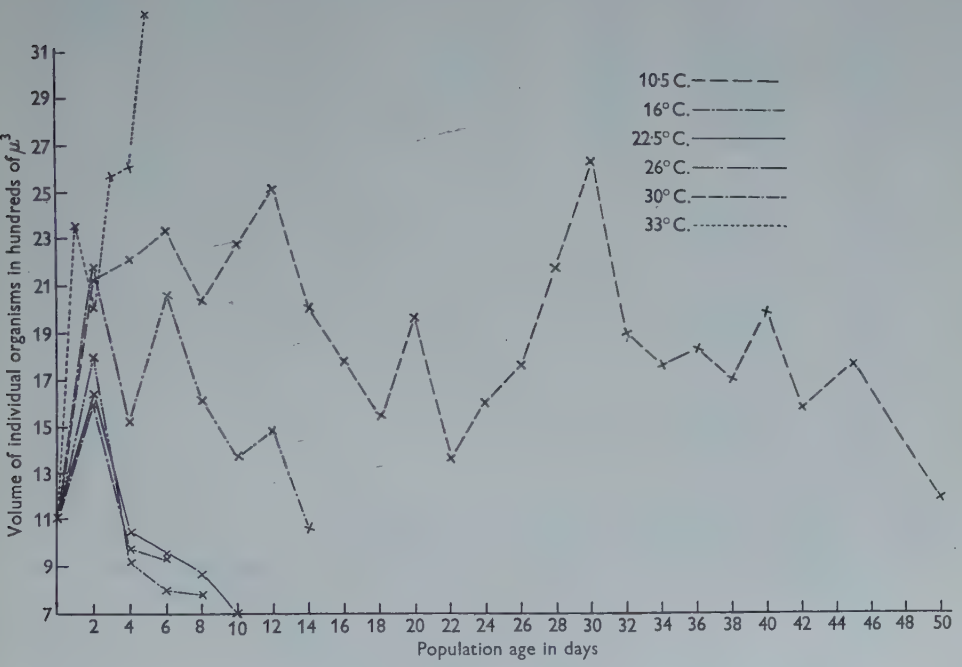

Fig. 6. Changes in volume of individual organisms of *Chilomonas* during population growth at different temperatures.

Since a sufficient number of good photographs was not always obtained from the same series (that is, from the same set of cultures from the same inoculum), it was of interest to follow changes in those series having the largest number of measurements from good photographs. These data appear in Table 9. Photographs of the cultures at 30° and 33° C. were all from the same series, so that the size data in Tables 5–8 for 30 and 33° C. are also from the same series.

It can be seen that the fluctuations with time, appearing at 10·5° C., also occur in one and the same series.

Fluctuations with time in the length of organisms at 10·5° C. are not accompanied by great changes in the coefficients of variation. These remain in the range 8·10–11·96% over a period of 30 days (Table 5). Fluctuations with time in the size and shape of organisms also appeared at 16° C. The reasons for these fluctuations at low temperatures is unknown.

Table 8. *Volume of* Chilomonas paramecium *at different temperatures and at different ages of population*

Days	10·5° C.	16° C.	22·5 °C.	26° C.	30° C.	33° C.
0	1,104	1,104	1,104	1,104	1,104	1,104
1	—	—	—	—	—	2,360
2	2,131	2,177	1,639	1,796	1,593	2,008
3	—	—	—	—	—	2,569
4	2,215	1,523	1,046	922	983	2,611
5	—	—	—	—	—	3,264
6	2,333	2,061	962	798	932	—
8	2,041	1,563	869	778	—	—
10	2,279	1,369	700	—	—	—
12	2,524	1,491	—	—	—	—
14	2,006	1,067	—	—	—	—
16	1,184	—	—	—	—	—
18	1,540	—	—	—	—	—
20	1,968	—	—	—	—	—
22	1,363	—	—	—	—	—
24	1,598	—	—	—	—	—
26	1,759	—	—	—	—	—
28	2,178	—	—	—	—	—
30	2,641	—	—	—	—	—
32	1,903	—	—	—	—	—
34	1,763	—	—	—	—	—
36	1,829	—	—	—	—	—
38	1,697	—	—	—	—	—
40	1,995	—	—	—	—	—
42	1,580	—	—	—	—	—
45	1,769	—	—	—	—	—
50	1,193	—	—	—	—	—

Table 9. *Size of* Chilomonas paramecium *at different temperatures. Data for each temperature from a single series of simultaneously inoculated cultures*

Days	10·5° C.					16° C.					22·5° C.					26° C.			
	L	*W*	*L*/*W*	*V*	*n*	*L*	*W*	*L*/*W*	*V*	*n*	*L*	*W*	*L*/*W*	*V*	*n*	*L*	*W*	*L*/*W*	*V*
2	25·3	10·5	2·43	1457	4	—	—	—	—	—	27·5	11·0	2·53	1742	2	24·0	12·1	1·99	183
4	27·7	11·7	2·38	1985	7	25·8	12·2	2·46	2010	13	22·6	9·3	2·43	1031	48	21·0	9·2	2·30	92
6	28·3	10·9	2·63	1760	8	28·5	11·5	2·50	1973	10	20·8	9·4	2·22	967	38	18·9	8·5	2·12	71
8	26·9	12·1	2·25	2041	22	26·6	10·6	2·54	1563	27	20·0	9·1	2·22	869	46	19·5	8·7	2·24	77
10	28·2	11·4	2·49	1918	10	25·7	10·1	2·57	1369	68	—	—	—	—	—	—	—	—	—
12	28·8	12·5	2·34	2355	19	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	29·3	11·4	2·58	2006	21	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16	27·7	11·1	2·52	1784	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	25·8	10·7	2·45	1540	28	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	28·3	11·5	2·47	1968	36	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22	26·6	9·9	2·73	1363	116	—	—	—	—	—	—	—	—	—	—	—	—	—	—

An interesting fact emerges from a comparison of the coefficients of variation of length, width and the ratio of length to width. If there is no correlation between length and width, then

$$E_r = \sqrt{(E_l^2 + E_w^2)},$$

where E_l, E_w and E_r are respectively the coefficients of variation of length, width and length/width. If the values of E_r, calculated from the above formula, are compared with the observed values as given in Table 7 it is found that in almost all cases (44 out of 48) the calculated value is greater than the observed value. This implies that there is positive correlation between length and width and therefore

that *under any given set of conditions* the shape of the organism tends to be preserved, whereas, as has been shown above, there are systematic changes in shape *as between one set of conditions and another.*

DISCUSSION

It is clear from these experiments, that *Chilomonas* can live and multiply over a wide range of temperatures—from 10·5 to 33° C. The genus belongs, therefore, to the eurythermic group of Protozoa. In his studies of factors influencing the distribution of ciliates in fresh water, Noland (1925) emphasized that most of them can live within wide temperature limits; and he added that such wide adaptability is to be expected, since they occur for the most part in shallow pools, where environmental factors are subject to great variation. This will also apply to the flagellate, *C. paramecium.*

The experimental results show clearly that populations of *Chilomonas* maintained at different temperatures differ in their growth rate but not in their maximum densities. It was known already from experiments on single organisms that temperature influences the division rate of Protozoa. Smith (1940) examined the effect of eight different temperatures on the division frequency of single *Chilomonas* kept in cavity slides for 24 hr. The range of temperatures corresponds to that covered in the present experiments, but as far as can be seen from his graph, the rate of multiplication of his organisms was lower. The optimum temperature lay between 26 and 30° C., and if the optimum is judged by the rate of multiplication, this corresponds quite well with the results of the present experiments, where the smallest generation time was recorded at 26° C., and was found to be slightly longer at 30° C. Regarding the growth curve as a whole, however, it would appear from the present work that cultures of *Chilomonas* thrive best at 16° C.; for while at 30° C. a few monstrous, partially divided forms appear as early as 6 days after inoculation, at 16° C. the organisms were still healthy in cultures 30 days old.

Gause (1931) made a mathematical analysis of the results of Terao & Tanaka (1928) on the growth of *Moina macrocopa* as an example of the effect of temperature on the size of population. Terao & Tanaka themselves used their results only to show differences in population growth at three different temperatures; Gause, however, not only drew a curve through these three points but gave its formula as well. It is questionable whether he was justified in choosing these experiments as an example, since *Moina* was taking protozoans as food, and we do not know the effect of temperature on the food organisms.

In the present study it was found that the maximum size of the population of *Chilomonas* does not depend, within wide limits, on the temperature. Graham-Smith (1920) found, however, that the largest size of bacterial populations, expressed in numbers of organisms, was reached at the lowest temperature. The life-span of the populations of *Staphylococcus aureus* was longest at 17° C. Monod (1924), working with *Bacterium coli*, also found that the total crop becomes progressively smaller with rise of temperature, being relatively constant between 29 and 33° C. and suddenly falling towards 40° C.

The present experiments confirmed for yet another biological system the variation of temperature coefficient (Q_{10}) and of μ (the thermal increment) with temperature: both decrease with increasing temperature. The coefficient b of Bělehrádek is relatively more constant, however, over a wide range of temperature. Phelps (1946) made use of the equations of van't Hoff and Arrhenius in interpolating values for growth rates at temperatures lying between two temperatures for which the growth rate is known; but such interpolation is clearly unjustifiable.

The change in size and shape of individuals at different temperatures was very striking, the organisms becoming larger and more slender at lower temperatures and smaller and plumper with rise in temperature; the plumpness at extreme temperatures (5 and 33° C.) was very marked. These changes in shape would seem to result from the fact that as conditions of culture are changed, change in length is not positively correlated with change in width; and it looks as if one and the same system in the cell is not responsible for growth both in length and in width.

Changes in size of Protozoa maintained at different temperatures have been observed by several workers. Popoff (1908) reported that the ciliate *Frontonia leucas* (like *Chilomonas*) was smaller at 25° C. than at 14° C. Rautmann (1910) also found that *Paramecium caudatum* became smaller with rise in temperature. Adolph (1929) observed that the size of the ciliate *Colpoda* decreased with increase of temperature from 17 to 26° C.; his suggestion that size is the quotient of rate of assimilation and rate of fission does not seem tenable.

Increase in size of ciliates at high temperature was shown by Scherbaum & Zeuthen (1954) in their experiments on *Tetrahymena pyriformis*. They exposed the organisms to a series of temperature shocks of short duration at 34° C., allowing recovery at 28° C. The organisms multiplied very little during the treatment, but became larger, as shown in their photographs. The authors abandoned Ephrussi's (1926–7) hypothesis, from which they started, namely, that the temperature coefficients, Q_{10}, are different for different phases of the mitotic cycle. They assumed that the sublethal temperature acted by blocking a specific step in the cell cycle; and referred to Phelps's (1946) finding, that at high temperature growth was reduced or did not occur at all. But this is a misunderstanding: Phelps's paper is only concerned with the population growth of *Tetrahymena*, while they were considering individual growth. They write: 'So we suggest that sublethal temperature prevents growth (e.g. synthesis), it also prevents new cells from entering a division, but it does not prevent a division, once initiated, from running to completion.' Their conclusion that the cells of *Tetrahymena* increased in size because they had been transferred to the 'optimum' temperature was incorrect; for experiments on *Chilomonas* have shown that a sublethal temperature does not prevent individual growth in this genus.

SUMMARY

Populations of *Chilomonas paramecium* have been maintained in a standard concentration of nutrient medium (0·1% beef-extract with 0·1% sodium acetate), at eight different temperatures, from 5 to 36° C. The temperatures 5 and 36° C. did

not support the growth of a population. The rate of population growth increased with increase of temperature up to a maximum, and then decreased. The maximum yield, on the other hand, was not affected, within wide limits, by different temperatures. The relationship between temperature and maximum size of population was different when maximum size was expressed in total number of organisms from that when maximum size was expressed in terms of total volume of organisms. In the former case, the maximum size increased with the increase of temperature; while in the latter it decreased with increase of temperature.

Bělehrádek's temperature coefficient, *b*, of the multiplication rate of *Chilomonas* did not show significant differences for different temperature ranges, while the coefficients Q_{10}, and the thermal increment μ were larger for the lower temperature range.

The size and shape of *Chilomonas* were found to depend on the temperature and on the age of the population. With increase in temperature, the size of organisms decreased to a minimum and then increased again, so that organisms were largest at extreme temperatures. In general, the organisms were more slender at lower than at higher temperatures. At all temperatures, the flagellate increased in size at the beginning of population growth and later decreased. Variation in size and shape was greatest at the beginning of growth and during transition to the maximum stationary phase.

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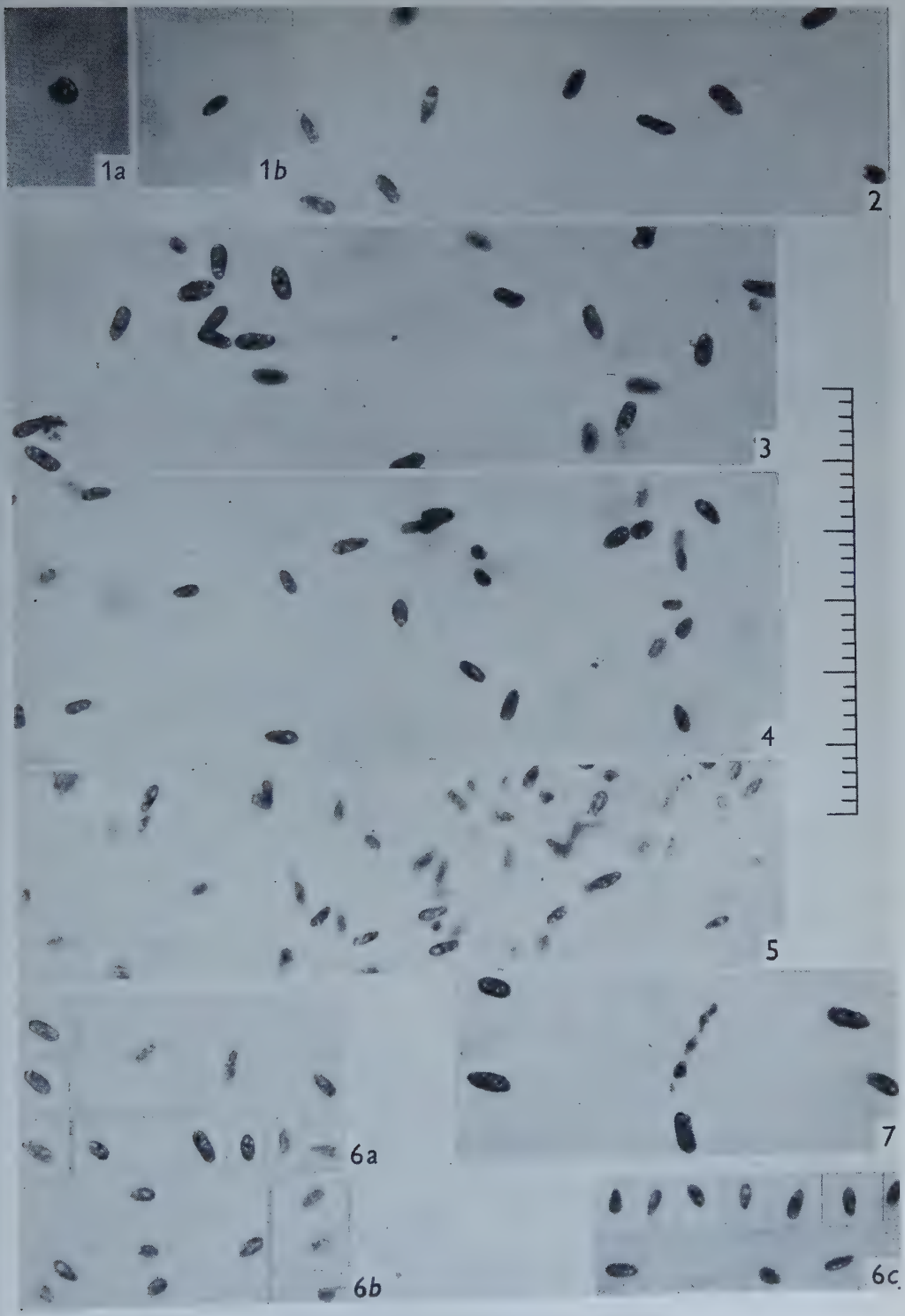
EXPLANATION OF PLATE 8

Chilomonas paramecium Ehrenburg: photographed alive without a cover-slip in cultures reared at seven different temperatures. Final magnification on plate: ×200. Fig. 1. 5° C., same individual in surface and edge view. Fig. 2. 10·5° C. Fig 3. 16° C. Fig. 4. 22·5° C. Fig. 5. 26° C. Fig. 6. 30° C., (*a*) population 2 days old; (*b*) population 4 days old; (*c*) population 6 days old. Fig. 7. 33° C.



SMILJA MUČIBABIĆ—SOME ASPECTS OF THE GROWTH OF SINGLE AND MIXED POPULATIONS OF FLAGELLATES AND CILIATES

(*Facing p.* 644)

ACRASIN, THE CHEMOTACTIC AGENT IN CELLULAR SLIME MOULDS

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In the life cycle of the cellular slime moulds *Dictyostelium* and *Polysphondylium*, the vegetative stage, in which separate amoebae feed on bacteria and multiply, is succeeded by a fruiting stage, in which the amoebae co-operate to produce a stalked spore mass; this stage is initiated when they begin to converge on certain collecting *centres* (Olive, 1902; Raper, 1940*a*, *b*, 1941; Bonner, 1944). Both Olive and Potts (1902), without offering any evidence, thought it likely that chemotaxis was involved in this orientation; and there the matter rested, except for a quite inadequate explanation involving negative hydrotaxis (von Schuckmann, 1925; Harper, 1926), till 1942, when Runyon showed that amoebae placed on one side of a cellophane dialysis membrane would duplicate the pattern of an aggregation that had formed on the other side; he concluded that a chemical agent was responsible. Bonner (1947), having pointed out that many types of agent would pass through cellophane, was in fact able by a great variety of experiments to exclude the influence of all other likely ones. More directly, he showed that when the amoebae were exposed to a slow current of water, they would approach a collecting point from downstream only while moving at random upstream of it. This made it probable that a chemical was indeed involved; but it might have been that the amoebae were reacting to a current when the chemical was present—a type of response so common, for example, among insects (Dethier, 1947). However, as in other experiments of Bonner's the amoebae were attracted in still water, it could be concluded that they were reacting to a chemical gradient. The evidence for the existence of the substance was sufficient to warrant Bonner's naming it—*acrasin*—though he was not able to isolate it *in vitro*.

Olive, influenced by Pfeffer's work (1884), tried to attract slime-mould amoebae with solutions of sugar and malic acid; fifty years later, Hirschberg & Rusch (1950) tried, with as little success, with echinochrome and dimethylcrocetin. Raper & Thom's demonstration (1941) that *Dictyostelium* and *Polysphondylium* grown in mixed cultures aggregated separately showed that the stimulus was highly specific and made it unlikely that chemicals used (or supposedly used) by other organisms for a similar purpose would be effective.

Pfützner-Eckert (1950) claimed that sensitive amoebae of *Dictyostelium mucoroides* would move towards a cell-free block of agar on which an aggregation centre

had rested; but I have not been able to repeat this experiment. However, it has proved possible to isolate acrasin *in vitro*, as has been briefly reported (Shaffer, 1953).

CULTURE METHODS

Dense cultures were obtained by inoculating slime-mould spores and *Aerobacter aerogenes* or *Escherichia coli* on agar plates rich in nutrient, prepared according to Bonner's (1947) recipe: after about 2 days at room temperature these furnished the acrasin sources used. The reactions of individual amoebae could only be studied in very much less populous cultures, which could be most easily produced by Singh's method (1946). The bacteria were grown separately on nutrient agar and then spread thinly on plates of $1\frac{1}{2}\%$ agar containing $0{\cdot}5\%$ sodium chloride adjusted to pH 6·5–6·8; these plates were inoculated with mould spores, and when all the food had been consumed there were sufficiently few amoebae present for agar to be visible between them. Sometimes the sodium chloride was omitted or replaced by a buffer.

EARLY EXPERIMENTS WITH NEGATIVE RESULTS

Pfeffer (1884) observed the orientation of plant sperm to the tip of a glass capillary tube containing a test solution; Went (1926) found that the amputated tip of an oat coleoptile could be functionally replaced in some respects by a piece of agar on which it had rested. These two methods are now classical, and many variations of them have been used in the present study in attempts to set up artificial gradients to which amoebae would react. The question of which amoebae are sensitive to acrasin is discussed in another paper (Shaffer, 1956*a*); here it may be said that they become sensitive at varying times before aggregation begins, so that sensitive ones can most regularly be obtained by thoroughly stirring up, with a glass rod, one of the *streams* into which the amoebae condense as they converge on a centre. The stream chosen should have been recently formed and still be flat, finely branched, and without any signs of developing swellings, which are liable to develop into secondary centres. The stirred cells may be rapidly carried on the end of the rod to any convenient site or swept there over the agar surface; they may be spread thinly or left in a streak or small heaps. Desiccation must be kept to a minimum. Raper & Thom (1941) found that *Dictyostelium discoideum* and *D. mucoroides* grown together would enter communal aggregations; and it has been shown that both these species and *D. purpureum* use the same acrasin for chemotaxis (Shaffer, 1953), so that test cells from these species may be used indifferently.

As for the acrasin sources, in experiments in which they have to be picked up with a glass needle, transplanted and sometimes bathed, it is easiest to use large, old centres, in which the cells are firmly packed. In *D. discoideum*, the aggregate migrates as an elongated slug before it begins to construct a fruiting body (Raper, 1935); this stage is the most convenient of all sources, because it secretes acrasin (Bonner, 1949) and is surrounded by a slime sheath, which protects it from damage when it is handled. Moreover, it tends to stay in contact with the water drop or agar on which it is placed: true, if conditions are too dry, it turns up its tip—which

is most active in acrasin production (Bonner, 1949)—into the air (Raper, 1940*b*), but this can be gently pushed down again. In other species, which lack the migratory stage, young fruiting bodies may be used as sources: they too are protected by a sheath; but they tend to leave the substratum immediately, and they are continuously being converted into non-secreting differentiated cells.

In the following tests the agar blocks were cut out with mounted rectangular fragments of razor-blades and placed on a culture plate 100–300μ away from sensitive amoebae, variously grouped: the tips of capillary tubes were arranged so as to lie near amoebae in the film of liquid on an agar surface or to touch the bottom of a dish containing sensitive amoebae under water, prepared by Bonner's method (1947). The test blocks and solutions were obtained as follows: sometimes, they were variously buffered from pH 4 to 8 (the limits for aggregation determined by Hirschberg & Rusch, 1950) with McIlvaine's at one-tenth of the standard concentration.

(1) A centre or migrant slug was removed, and the agar under it swept clear of cells and cut out.

(2) Small blocks of agar from 100μ to several millimetres thick and of area just greater than that of the slugs to be placed on them were loaded in a saturated atmosphere with from 1 to 50 slugs (groups 10^5–10^6 times smaller constitute an effective natural source). The slugs were left there for 1 min. to 1 hr. and then the blocks were carefully cleaned of cells.

(3) From 1 to 50 slugs were bathed for 30 sec. to 30 min., in a saturated atmosphere, with a drop of water that formed a barely visible layer or extended up to 500μ from their edges. The slugs were not more than half immersed, for if they were completely covered they rounded up and degeneration began; this was in line with the findings of Potts (1902) and Bonner (1947) that aggregation but neither migration nor fruiting would occur under water. The continued healthiness of a slug was recognized by its remaining elongate with a sharply defined tip. The water was collected in capillary tubes or absorbed on dry agar blocks or fragments of washed filter-paper.

(4) Slugs were sucked up a capillary pipette, with the minimum of water, and heated to 50° C. for a few minutes. The contents were tested as before, or the dead slugs were expelled on to agar near sensitive cells. Other slugs were picked up with a needle, brought up to a block of solid carbon dioxide and replaced, dead, on the agar.

In no case were amoebae found to collect at the artificial sources or to orient preferentially to them.*

Two possible explanations were available to account for these negative results. First, the living sources might have imposed additional characteristics on the chemical signal. Arndt's time-lapse film (1929, 1937) showed that aggregation was rhythmic in *D. mucoroides*; and it might have been that acrasin had to be delivered in pulses. However, though Bonner's film (1944) of *D. discoideum* showed that waves of rapid inward movement did pass over some aggregations, they did not do so in every case. Moreover, the pulses were produced at intervals of about 5 min.

* Dr J. T. Bonner has since told me that he too had no success with similar sources.

and appreciable movement occurred between them; so one might have expected an artificial source, providing a single pulse, to have stimulated readily detectable orientation, even if only temporarily. The other possible explanation was that the secreted chemical rapidly disappeared because of inherent instability or active destruction or volatility, though the last was improbable as it would not have explained the results obtained with capillary tubes.

THE THEORY OF CHEMOTAXIS

A single molecule, continually buffeted by those of the medium, pursues an irregular and random course; so that from its direction at any moment, it is not possible to learn whence it has come whatever receptor organs are available. When a directional element is not provided by a current or the movement of the source itself, this can only be located by sensing the distribution of the chemical; and since the position of each molecule is determined by chance, the judgement must be based on sufficient numbers to make it probable that the direction in which the concentration increases most steeply is one that leads towards the source. This limits the possible sensitivity. As each of them functions as one of a crowd, the molecules used should, on grounds of economy, be as small as is consistent with specificity.

It might be expected that the least difference of concentration, Δc, detectable by either simultaneous or successive comparison, would not be constant but would be related to the average concentration, c, at the points of comparison. If $\Delta c/c$ were constant, that is if the Weber-Fechner Law were obeyed—as Bonner and Savage (Bonner, 1947) assumed that it was—the maximum distance from which an amoebae could be attracted to a point source would be independent of the absolute amount of any stable chemical that was secreted, for this does not effect its distribution. Even if $\Delta c/c$ were large when c was small and tended to fall to a minimum value as c increased, the range at which the source could be detected would tend to reach a maximum as more chemical was produced.

This limiting range could be extended if the chemical were removed some time after its emission. This may become clearer if the outputs by a source during successive minutes are considered separately—legitimate if the chance of a molecule's inactivation is unrelated to the concentration. The molecules secreted during a given minute would at first be crowded together near the source; then they would move outwards, tending to an equal distribution (zero concentration) everywhere. The concentration pattern the cells would experience would be the summation of the outputs of all the minutes. It is clear that if the chemical were removed in a first-order reaction, the fraction of molecules left from earlier outputs, which would have a flatter distribution, would be less than from the later ones. As a result, the summated relative concentration gradient would become greater, though the concentration would be reduced. Provided, then, that the source could increase its output sufficiently, cells of a given sensitivity would be attracted from a greater distance, even in an infinitely extensive medium, if the active chemical disappeared. If the limits of diffusion—whether set by the boundaries of the medium or the

presence of other sources—were near to the source, the removal of the chemical would become not merely a means of extending the range but a necessity in order to maintain, for long periods of time, any effective range at all. If it were not removed, the gradient produced by the secretions of later minutes would have to be detected against an obscuring background of ever-growing strength. The signal could indeed be strengthened by greater secretion, but as this would be converted into 'noise', the benefit would be short-lived; and the final situation would be worse than before, because the accumulation of the chemical in the medium would depress its secretion by the source.

However, it would be possible to extend the period during which a source could guide cells in a limited environment using a stable chemical if this were released in short pulses, the intervals between them during which the gradient would be undetectable being of such a length that the cells would still be moving predominantly in the direction in which they had been oriented by the last pulse when they again received direct guidance from the next one. Such an arrangement would also make the most efficient use of the chemical produced, whether it were stable or not.

These considerations pointed to one or both of the same conclusions as did the last section, namely, that acrasin rapidly disappeared from the external medium and that it was released in pulses.

THE ISOLATION OF ACRASIN

If either conclusion was correct, sensitive cells might only react to an experimental source if it could be renewed at frequent intervals. It was clear that such a source would have to be a liquid rather than an impregnated agar block; the difficulty was that if the sensitive cells were under water, each addition of liquid would upset any diffusion gradient, and if the cells were on agar, the added liquid would not remain localized and reinforce the gradient but would simply spread over the surface. This was overcome by sandwiching the sensitive cells between an agar block and a glass slide; liquid could then be added repeatedly to the meniscus round the outside of the block without its mechanically disturbing any diffusion gradients inside. With this method it was possible to renew the artificial source as often as every 5 sec. with material taken from the vicinity of the natural source only 2 sec. earlier.

The moist chamber used had glass slides for top and bottom and an opening at one side through which glass instruments could be introduced. With a micropipette, a number of water drops, some of about 10^{-2} ml. and others of 10^{-3} ml. or smaller, were deposited on the lower slide, leaving a central area clear of all but two small drops, 5–10 mm. apart. With a glass rod, the drops could be moved about on the slide, if this was not too clean, and smaller droplets detached from them as needed. Migrant slugs of *D. discoideum* were transferred, individually with a needle, to one of the central drops; they were placed parallel to each other but separated. The test cells had been grown on saline agar 0·5–1 mm. thick. A small block of it, about 2–3 mm. square, carrying at least part of a flat stream or of an early finely branched aggregation from which the centre had been removed, was cut out. The block was

picked up on the razor blade, turned upside-down, and gently eased off with a glass rod into the second central droplet of water. With care, at least part of the stream was trapped between block and slide and could be brought into any desired relationship with the edge of the block: having regard to Bonner's (1947) figures for the greatest distance from which cells could be attracted to a natural source, the preferred position of the stream was parallel to and about 150–200 μ from the edge. The block was cut with slightly sloping sides, the surface above after inversion being rather larger than the lower one: this made it possible to observe cells on both sides of the edge, when there was a little liquid round the block.

The water bathing the slugs was changed till it contained no more loose cells, and only enough of it was left to extend halfway up each slug and 50–100 μ away from its side. Then the block was gently pushed to and fro, after first removing almost all the water around it: this completely disrupted the stream and ensured that the amoebae were unoriented but free to respond. After a delay of 2 to 3 min., which allowed time for the return of motility but not of intercellular organization, the transfer of liquid from the slugs to the edge of the block was begun (Fig. 1). About 10^{-5} ml. from round the nearest slug was carried over in a pipette, or dragged across with the bent tip of a glass rod, and then replaced with water from one of the drops on the floor of the chamber; at 10 sec. intervals, further transfers were made from successive slugs. Thus, with a row of thirty slugs, each was allowed to secrete acrasin into the surrounding water for at least 5 min. before this was collected. If the experiment was lengthy, it was necessary to remove some of the liquid accumulated round the agar block to prevent excessive dilution of the later transfers.

Usually, the amoebae could be seen, within 5–10 min., to be oriented perpendicularly to the edge; they first began to reach it after 20–30 min. or more, depending on the distance they had to travel. If they were not started too far away, they were still more or less parallel to each other on their arrival there. The orientation of the amoebae agreed with expectation, since, if acrasin were present in the liquid at the edge of the block, it would be rather evenly distributed through it, and the internal gradient would be normal to the surface.

It was known (Shaffer, 1956*a*, *b*) that the leading end of an undisturbed stream fragment, or of a reorganized group of amoebae from a stirred-up stream, might not advance at all even in the presence of an acrasin gradient, or that it might do so even in its absence. Moreover, a small proportion of the amoebae from disintegrated streams might move at random in such a gradient. So, for example, in a test, a stream fragment might reach the edge of the agar block by chance and yet simulate one that had been attracted there; its front end, once escaped from under the block, would almost never find its way back, and the rest of it would continue to grow by collecting sensitive amoebae. Because of these possibilities, the criterion that an acrasin gradient was present if and only if amoebae crawled towards the edge was too simple: attraction to the edge was considered positive only if a large number of moving amoebae able to make independent judgements of the gradient agreed in their orientation. If most of the amoebae did not move, the experiment had to be abandoned.

Positive results could still be obtained even if the length of time the water bathed the slugs and the frequency at which the drops were transferred were both much reduced. It was found convenient to use two, or only one, small groups of slugs for supplying acrasin solution and to transfer rather larger drops of it to the block once a minute. No positive results were obtained if no liquid was added to the edge or if untreated water only was added whatever the frequency.

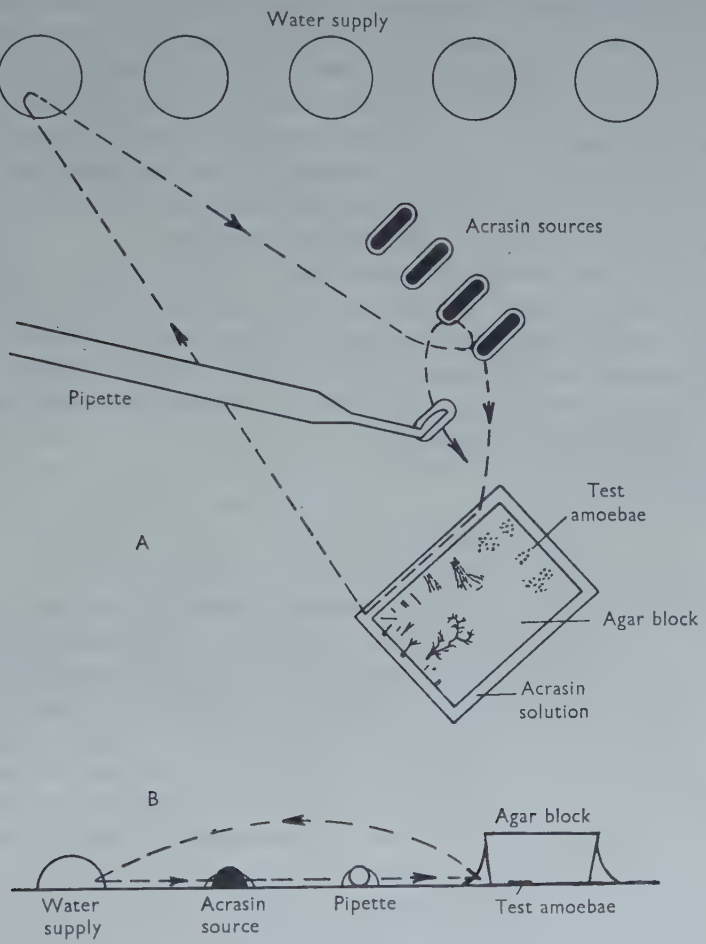

Fig. 1. Plan (A) and elevation (B) of the layout for the agar-block test for acrasin. In A, the test amoebae under the block are represented on one side as unoriented and on the other as having responded in various ways to the artificial gradient.

However carefully the slugs were handled, it was difficult to ensure that not a single loose cell was present in the washings; though it was unlikely that the very few that were transferred to the block were responsible for the attraction, because the test amoebae moved parallel to one another, whereas when a natural source was placed against the edge, they converged on it.

To eliminate all loose cells, the washings had to be filtered: each drop was

deposited on the upper surface of a small piece of Millipore membrane, about 5 mm. square, to which a wax border had been applied; with another pipette, it was collected from underneath it a few seconds later and transferred to the agar block. The amoebae oriented to the cell-free source.

It was possible that asymmetrical addition of washings to the block produced underneath it a slight slow water current and that it was this, when it carried acrasin, that stimulated orientation. To investigate this, a block was selected that had under it at least some sensitive amoebae near each of its sides. To minimize any currents set up as a result of its absorption of water during the subsequent test, it was first soaked in water. After the excess had been removed, the upper surface was covered with a fragment of cover-slip to prevent evaporation from it. When acrasin solution was added, repeatedly, as evenly as possible all the way round, each group of amoebae crawled outwards perpendicularly to the side closest to it, except for those near the corners, which adopted an intermediate position.

The possibility that the amoebae were led to the edge by reacting to a gradient of some sort produced by their own metabolism, which they did only when activated by acrasin, could be ruled out by working with a long and very narrow block under which the amoebae were positioned to one side of the mid-line: if plain water was added to the edge nearer to them and acrasin solution to that further away, the amoebae crawled towards the latter.

It was concluded that the amoebae were guided by a gradient of acrasin.

THE REACTIONS TO AN ARTIFICIAL GRADIENT

Amoebae confined under a slab of agar showed a variety of behaviour patterns; but as these were much the same as those occurring in other environments (Shaffer, 1956*b*), they will not all be detailed here. Separate sensitive amoebae were guided by the artificial source as already described; and, if started near enough to the edge, they were still in parallel formation as they crawled out from under the block. Usually this pattern was distorted, because the oriented amoebae soon began to attract their neighbours. If there were rather few amoebae present, they lined up, mainly end to end in single file, to form parallel threads, which entered minute but growing clumps at the edge of the block. As more amoebae joined in, each thread became a delicately branched aggregation. Groups of amoebae were drawn out into arrowheads aimed at the source. The closer the amoebae were to each other and the further they started from the edge, the greater the opportunity they had to interact, producing numbers of variously branched streams. The front end of each of these was guided by the resultant acrasin gradient, whether this led it to the edge of the block, to the neighbouring streams on its flanks, or even back to the cells following it, in which case a ring was produced; alternatively, it simply stopped moving and formed an independent and perforce very flat centre. At times, some or all of a stream disintegrated: its constituent amoebae separated from each other and then reoriented independently to the gradient. Though streams that did reach the edge of the block had no gradient to guide them further, they crawled on for varying

distances, because of their inherent motility, and then began to pile up in stationary heaps. As a result of the continual disturbance of the water, these usually became detached from the glass.

Sensitive amoebae, that might or might not have previously been in an aggregation but had not aggregated after more than an hour under an agar block, would almost at once begin to form branching and mutually attractive streams when exposed to acrasin solution: this showed that acrasin stimulated them to secrete acrasin.

THE INSTABILITY AND STORAGE OF ACRASIN

It was now necessary to consider why the earlier tests with artificial sources had failed. An explanation exclusively in terms of the need for releasing acrasin in pulses became unlikely: positive results had been obtained with transfer frequencies varying from 10 sec. to 2 min.; moreover, films of the agar-block test showed cells moving smoothly towards the edge without waves or pulsation.

The alternative explanation in terms of instability was investigated by detaining the acrasin solution during transfer: it was collected every minute with one of a number of micropipettes used in rotation; it was delivered to the edge of the block after a delay of as many minutes as there were pipettes. The very large number of variables involved in collecting and testing made quantitative assay extremely difficult; nevertheless, it was established that, in general, solution stored 5 min. was weaker than that stored but 1 min. or not at all, that stored 20 min. only rarely had any detectable effect, and that held longer had none.

Whereas the single application of fresh solution, which set up a gradient continuously declining in strength, did not excite perceptible orientation, the repeated addition of solution 10 min. old might do so after about 10 min. Despite this finding and the advantages of releasing acrasin in pulses discussed above, to which must be added that of enabling a chemotactic relay system to function in an orderly way (Shaffer, 1956*c*), there was still nothing to suggest that the single pulse produced by an unrenewed artificial source, provided that it was strong enough for long enough, could not cause easily detectable even if temporary orientation.

It seemed likely that acrasin would be considerably more stable at a lower temperature. In order to test this, each drop of solution, after filtering through Millipore, was collected with a separate micropipette, which was then immediately dropped into one of a series of holes in a brass block cooled by solid carbon dioxide. To examine the potency of the solution, a pipette was withdrawn each minute, and its contents, after thawing, added to the edge of an agar block, as in the regular test. The operations on the acrasin solution—collection and filtering, transfer to the brass block, freezing, thawing, and transfer to the agar—together took less than a minute. A solution thawed after several days in the cold was still very active in causing the orientation of sensitive amoebae, though it rapidly lost its activity at room temperature. That temperature gradients were not responsible was shown by freezing the solution after it had been kept at room temperature for an hour and then testing it, as before, after thawing: no orientation was induced.

In an experiment to confirm Runyon's finding (1942) that the stimulus to aggregation would pass through cellophane dialysis membrane, slugs were grouped in a drop of water on a small raft of this material. Every few minutes, water was added underneath the cellophane and then collected again: it oriented amoebae in an agar-block test. It was found to differ from the cell-free acrasin solution obtained by filtration through Millipore in being stable at room temperature. It was concluded that acrasin activity was associated with molecules small enough to pass rapidly through cellophane and that loss of activity was due to reaction with much larger molecules, possibly of an enzyme, also present extracellularly.

CHEMICAL EXTRACTION

An attempt was then made to extract acrasin chemically. The method adopted was to pour absolute methyl alcohol, cooled on solid carbon dioxide, on to a phosphate-buffered culture plate in which aggregation was general. After 4 min. in a cold-room, the liquid was poured off and dried *in vacuo* below −10° C. The residue was extracted with a small volume of methyl alcohol, which was then filtered and again dried. An aqueous solution of the product, which had a pH of about 5 and was strongly buffered, had a very high acrasin activity. Activity was retained after boiling for more than an hour and after exposure to excess N/10 hydrochloric acid or N/100 sodium hydroxide. Dry acrasin was considerably less soluble in absolute ethyl alcohol than in methyl.

A cell-free aqueous extract of an aggregating culture had the power to inactivate acrasin but lost it after it had been boiled: the fraction that could be precipitated with half-saturated ammonium sulphate was shown to be responsible. This knowledge suggested a more convenient procedure for obtaining acrasin. Agar bearing aggregations was simply dropped into boiling water, the solution evaporated, and the residue extracted. Acrasin was collected in a rather purer form by continuously dialysing it away from its attacker during aggregation.

A single addition of a concentrated acrasin solution to the edge of a test agar block produced dramatic orientation of the amoebae within 2–3 min.: they could have been exposed to only a single pulse from the added acrasin.

DISCUSSION

Acrasin (named or unnamed) has been sought, on and off, for more than half a century. Its elusiveness may have frustrated the research worker; but the instability that has been found to lie behind it is most probably of value to the slime mould in enabling it to maintain effective guidance for its amoebae. The discovery that the amoebae produce a protein—probably an enzyme—that inactivates acrasin extracellularly tends to support the view that this action is of use. Separated from its attacker, acrasin is a remarkably stable molecule; but apart from this, there is still little known of its nature, other than its being soluble only in water and solvents very much like it.

When and where the inactivator is produced has not yet been investigated; but however its secretion is related to that of acrasin's, it would be extremely costly in time and material for a source to set up a detectable relative concentration gradient of acrasin at a considerable distance from itself. In fact, the maximum range from which amoebae can be directly attracted is about 350μ (Bonner, 1947)—the sort of distance one might have expected for a process based on diffusion. With a series of relays each covering this distance, it would be possible to increase the total range more or less indefinitely, both cheaply and quickly. Such a system does indeed exist, based on the action of acrasin in stimulating amoebae to secrete acrasin, and it permits an aggregation to extend for several centimetres.

When thousands and even hundreds of thousands of separate acrasin sources are present in a single aggregation, the problem of maintaining detectable gradients is vastly aggravated. To see what effect the inactivation might have, we must make a number of assumptions; and as these have not yet been tested, an exact treatment is scarcely called for. If the inactivator is secreted by all the amoebae and is comparatively stable, we may expect it to be more evenly distributed than the acrasin. If, as a first approximation, we assume that it is present in the medium at the same concentration throughout the aggregation area, that it is an enzyme, and that Michaelis kinetics are obeyed, the reaction velocity of inactivation will be roughly proportional to the local acrasin concentration when this is well below that corresponding to the Michaelis contant and will tend to a limiting value when it is much higher. Thus, inactivation of acrasin by an enzyme will be at least as efficient as that by a first-order reaction, which was discussed above: at low concentrations of acrasin it will approximate to the latter, and at high ones it will be better, in that, in effect, it will tend simply to reduce the background against which the gradient has to be detected.

Though the release of acrasin in pulses has been shown not to be an essential of the orientation mechanism, it may none the less be of value, partly in facilitating the maintenance of a gradient, partly in economizing the chemical (if this is of importance to the organism), and it is quite probably involved in the rhythmic movements during aggregation photographed by Arndt (1929) and Bonner (1944).

The problems that slime moulds have faced in using chemotaxis in morphogenesis are further considered in another paper (Shaffer, 1956*d*).

SUMMARY

1. A study has been made of acrasin, the agent inducing chemotaxis in the amoebae of cellular slime moulds.

2. A method has been developed for subjecting sensitive amoebae to a fluctuating gradient set up by an artificial source that can be renewed at intervals of as little as a few seconds with fresh test solution.

3. Amoebae orient to a gradient maintained with the cell-free liquid freshly obtained from the immediate surroundings of a natural source.

4. Acrasin solution as secreted loses its activity very rapidly at room temperature.

5. A highly active stable solid is obtained by drying methanolic culture extracts; it resists boiling and exposure to acids and alkalis. Its solubility decreases rapidly in passing up the alcohol series.

6. The original instability has been shown to be due to the presence of another extracellular slime-mould product, possibly an enzyme; it, unlike acrasin, cannot pass rapidly across a dialysis membrane, is heat labile, and can be precipitated by ammonium sulphate.

7. The advantages of the organism's itself inactivating acrasin are considered.

8. Some of the advantages of a source's releasing acrasin in pulses are discussed; but it is not essential for orientation for it to do so.

9. Sensitive amoebae not only are oriented by an acrasin solution but are caused to secrete acrasin: this is the basis of a chemotactic relay system.

I should like to thank the members of the Department of Biology, Princeton University, for their hospitality during my tenure of a Dill Fellowship, especially Dr J. T. Bonner for his, and for his interest in this work, and Dr F. I. Tsuji for his help. To Dr B. N. Singh I am grateful for sending me cultures, and to Dr M. G. M. Pryor I am deeply indebted for his continuing encouragement.

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PROPRIOCEPTION IN *LIMULUS*

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(With Plate 9)

INTRODUCTION

Perception of the mechanical stimuli resulting from movement of Arthropod limbs has now been shown, in a number of cases, to involve special proprioceptive sensory endings at the joints. Apart from the internal organ found by Burke (1954) at the dactylopodite joint of the leg of *Carcinus*, these sense cells send peripheral processes which terminate in or directly under the cuticle, and the immediate stimulus appears to be the mechanical strain set up in the cuticle near the hinge by joint movement or by forces acting more distally on the leg. In Crustacea (Tonner, 1933) processes of the sense cells terminate without secondary cuticular modification in the joint membrane, but in insects (Pringle, 1938*a*) and in scorpions and *Phrynichus* among the Arachnida (Pringle, 1955) cuticular structures are associated with the endings in the form, respectively, of campaniform sensilla and lyriform organs, and serve to define more precisely the nature of the mechanical stimulus to which the endings respond. There are differences in structure between the campaniform sensilla of insects and the lyriform organs of Arachnids which indicate that these two types of sense organ have had a parallel rather than a consecutive evolutionary history, but it is probable that they serve an analogous function in these two major groups of terrestrial Arthropods.

The campaniform sensilla of insects and the lyriform organs of scorpions and *Phrynichus*, when suitably excited, initiate a discharge of impulses in their sensory nerves which shows slow and incomplete adaptation, and these endings can therefore provide continuous information about the position of the leg joints or the forces occurring during movements of the animal. In the cockroach many or all of the groups of campaniform sensilla on the legs are so situated that they signal the forces produced by contact of the leg with the ground rather than the position of the particular joint, and another type of sense organ, the hair plates (Pringle, 1938*b*), is probably the more important indicator of joint position. In scorpions movement of the joint is capable of eliciting a large sensory discharge even when the leg is out of contact with the ground, the impulse frequency in most of the fibres from the lyriform organ depending on joint position, with some acceleration of discharge during actual movement and if movement is resisted by an external object. In addition, there are present in the legs of the scorpion and *Phrynichus* sensory

endings with fibres of large diameter which are excited only by rapid movement of the joint in one direction and which adapt rapidly and completely when movement ceases. Certain characteristics of these discharges suggested (Pringle, 1955) that they did not originate from lyriform organs in the cuticle but from some form of internal ending at the joint whose nature was not then clear.

In a comparative survey of proprioception in the Arthropoda it is clearly important to include *Limulus*, an archaic, bottom-living, marine animal related to the stock from which the terrestrial Arachnida are descended. Opportunity to make a study of this sense in *Limulus* occurred during a short visit to the Woods Hole Marine Biological Laboratory in August 1955. After the experiments here described were completed it was discovered that Barber (1954) and Stuart (1953) had already interested themselves in this problem and some results have now been published by Barber (1956). Mr R. W. Stuart, whose histological work forms a valuable counterpart to the present experiments, has kindly allowed me to redescribe his observations in this paper and to publish some of his photographs of histological preparations.

MATERIAL AND METHODS

Large adult specimens of *Limulus polyphemus* (L.) were kept in the laboratory tanks and the legs removed as required by amputation across the base of the coxopodite. No differences were found between results from different legs.

Impulses were recorded in the leg nerves by means of wire electrodes, a Grass P4 Pre-amplifier and a Grass Oscilloscope Camera.

To produce controlled mechanical movements, usually at the femoro-tibial (ischiopodite-meropodite) joint, the basal segments of the leg were firmly pinned to a wax block and the middle of a thread tied to the distal segment. One end of the thread was then attached by a length of elastic to a fixed support and the other wound on the shaft of a potentiometer controlling the Y-shift of the second beam of the oscilloscope. Approximately linear indication of joint movement is thus presented on the record.

RESULTS

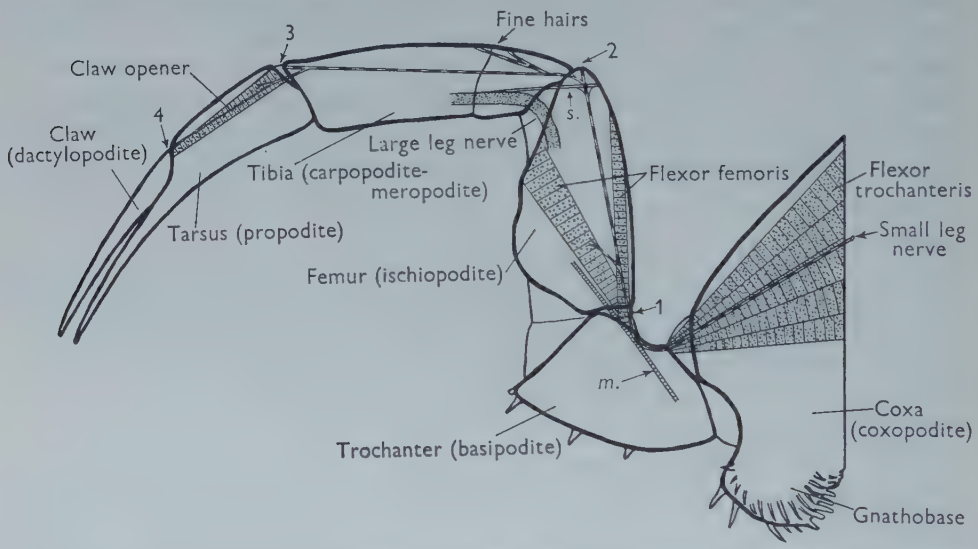
Anatomy and histology

Attention has been concentrated on the small leg nerve (the external pedal nerve of Hanstrom, 1928), whose field of innervation is shown in Text-fig. 1. This nerve arises independently from the ganglion ring and contains motor fibres to only three muscles, the flexor trochanteris in the coxa, the flexor femoris in the femur and the opener of the claw in the tarsus. The first two of these are levator muscles in normal locomotion and lift the leg off the ground; as in many Arachnids there is no levator muscle (extensor) at the femoro-tibial joint, this movement being brought about by a positive blood pressure and failing after excessive bleeding (cp. Ellis, 1944).

Only a small area of the leg surface on the outside of the tibia (near the line of fusion of the carpopodite and meropodite) has its tactile innervation in the small leg nerve. By far the largest and most noticeable sensory fibres arise from groups of

cells lying near the joint membranes at the trochantero-femoral, femoro-tibial, tibio-tarsal and claw joints (1, 2, 3, 4 in Text-fig. 1). These cells were first described by Stuart (1953), whose histological results are now quoted in detail. Similar cells at the coxo-trachanteral joint, which are innervated by one of the gnathobase branches of the main leg nerve, have been described by Barber (1954); these have not been studied in the present investigation.

Stuart's results were obtained with methylene-blue staining of the leg nerves. He found groups of sensory cell bodies under the median membranes of the first and second joints and under the lateral membranes of the third and fourth joints.



Text-fig. 1. The right third leg of *Limulus*, showing the field of innervation of the small leg nerve. 1, 2, 3, 4, location of proprioceptive endings; *m.*, special muscle (see text); *s.*, connective tissue strand.

The cells are typically multipolar with a single axon leading to the nerve and branching processes ramifying peripherally into the hypodermal cell layer under the joint membrane (Pl. 9, i, ii, iii). The size of the cells varies from $30 \times 60\mu$ to $45 \times 100\mu$, with indications of two distinct classes, a larger and a smaller. Differences are regularly found between the size of the cells at different joints. Thus, the groups at the median inter-segmental membrane of the first (coxo-trochanteral) joint contains a group of ten to fifteen cells of about 40×80–90μ and, more distally, another group of smaller cells about $30 \times 60\mu$, with thinner peripheral processes. The cells at the second joint (trochantero-femoral; 1 in Text-fig. 1) form a single diffuse group all of the smaller class ($30 \times 60\mu$). At the third joint (femoro-tibial; 2 in Text-fig. 1) a group of very large cells, 45×90–100μ, are found 'at some distance from the lateral intersegmental membrane on the sensory nerve innervating this membrane and close to the junction of this nerve branch with the external pedal nerve' (Pl. 9, i); there is also a more numerous group of smaller cells 'distributed throughout the hypodermal tissue of the third joint's lateral membrane'.

Dissections made during the present investigation revealed that there are two short branches of the small leg nerve innervating cells at the femoro-tibial joint. The small cells giving rise to these sensory axons lie in two groups on each side of the hinge line, one group being thus at the extreme distal end of the femur (ischiopodite) and the other at the extreme proximal end of the tibia (meropodite).

Stuart also found two closely adjacent groups of cells at the fourth joint (tibiotarsal; 3 in Text-fig. 1) and his illustrations of these are reproduced in (Pl. 9, ii, iii). The cells of Pl. 9, iii belong to the smaller class, $30 \times 60\mu$, with fine peripheral processes, and those of Pl. 9, ii to the larger class, 40×80–90μ, with thicker peripheral processes; the branching of the processes in the hypodermal cell layer is well displayed.

Physiological evidence was obtained in the present investigation for endings in the claw joint (4 in Text-fig. 1).

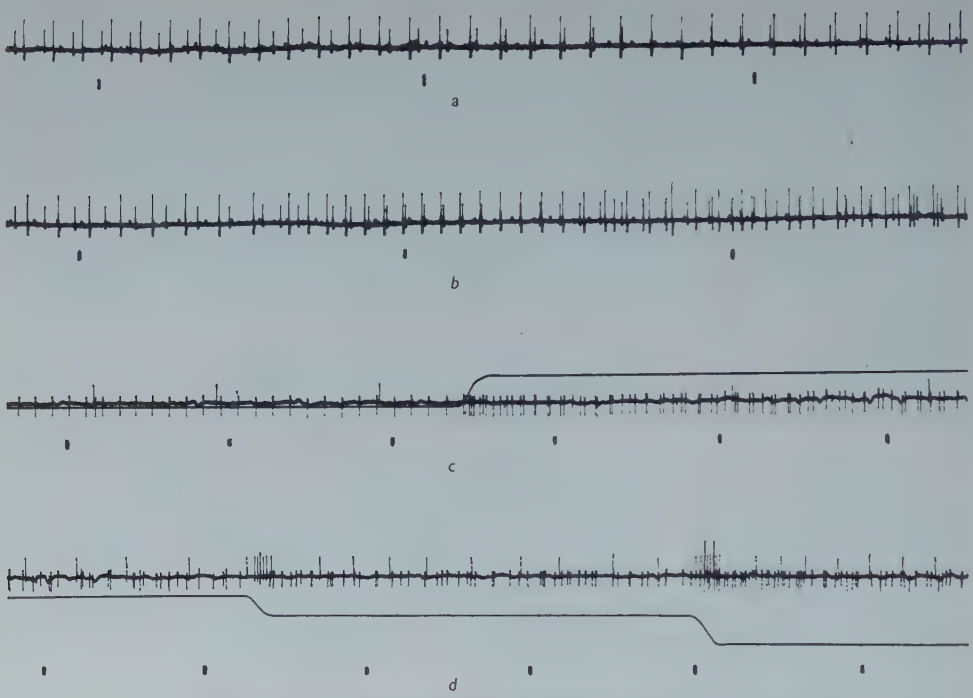
Physiology

Recordings have been made of impulses in the small leg nerve at two positions: in the nerve exposed in the femur and at the extreme base of the coxa. At the base, fibres are present sensitive to movement of the trochantero-femoral, femoro-tibial, tibio-tarsal and claw joints. The largest impulses were obtained from endings at the femoro-tibial joint and, since these appeared to be typical of the leg proprioceptors, they only were studied in detail.

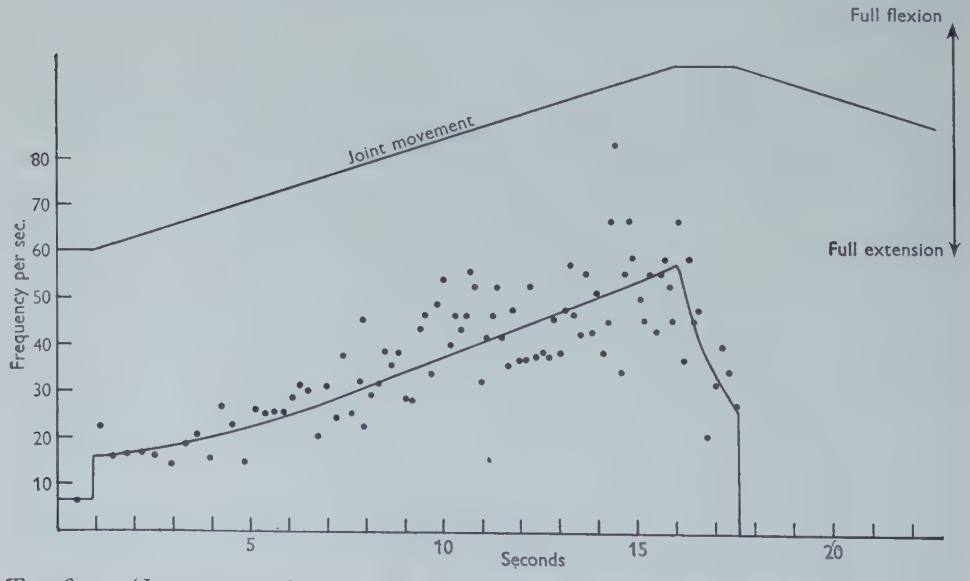
Text-fig. 2*a* shows the extremely regular discharge of impulses which may be obtained from endings at the femoro-tibial joint when this is held steady in a position of partial flexion. The frequency in these two fibres is, respectively, 10·7/sec. and 11·1/sec. and the discharge continues indefinitely. Allowing the joint to flex under the natural elasticity of the flexor muscles increased these frequencies to 13·9/sec. and 14·5/sec. and brought in a third fibre at 6/sec. (Text-fig. 2*b*); impulses in many smaller fibres were visible with high amplification.

There are present at the femoro-tibial joint tonic (incompletely adapting) endings whose discharge frequency increases on extension as well as endings excited by flexion. Text-fig. 2*c* shows such an ending responding to forced extension, in the middle range of joint movement, produced by the thread of the joint-position indicator; Text-fig. 2*d* shows two forced flexions in the same preparation. In Text-fig. 2*c* at the initial position of the joint an extensor fibre is discharging at 9·5/sec. and a larger flexor fibre at 1·15/sec.; at the final more extended position the extensor fibre, after an initial acceleration, is firing at 13/sec. and is joined by another of similar amplitude while the flexor fibre, after a short silence, returns at 0·85/sec. In Text-fig. 2*d* several flexor fibres are active and their frequency of discharge rises as flexion is increased; the largest of the tonic fibres shows considerable acceleration during movement and the second flexor movement initiates also two impulses from a phasic ending which has the largest sensory fibre in the whole nerve trunk.

A graphical plot of the response of the largest tonic flexor fibre to slow flexion is shown in Text-fig. 3. It is clear from the original record that many of the irregularities of frequency in this experiment resulted from failure to produce an exactly



Text-fig. 2. Oscillograms from *Limulus* small leg nerve, showing impulses in fibres from proprioceptor neurons at the femoro-tibial joint. *a*, joint partially flexed; *b*, joint allowed to flex fully under elastic pull of flexor muscle; *c*, nerve exposed in femur, discharge in tonic extensor fibres on forced extension, inhibition of tonic flexor fibre; *d*, nerve exposed in femur, discharge in flexor fibres on forced flexion, starting from more flexed position than record *c*. The second movement brings in a phasic fibre (retouched). In records *c* and *d* an upward movement of the joint-position indicator trace signifies extension. Time marker, 1 sec.



Text-fig. 3. 'Instantaneous frequency' plot of discharge in a tonic flexor fibre from femoro-tibial joint during slow joint movement. Each point is the reciprocal of the interval between one impulse and the next, and is referred to the instant of time mid-way between them. This ending was initially firing at 7/sec.

constant velocity of joint movement with the hand-operated control knob of the potentiometer (see Methods). This is the same flexor fibre whose response to more rapid movements is shown in Text-fig. 2*c*, *d*; the complete silence during even slow extensor movement shows that there is a considerable phasic influence in the excitation of this ending.

The overall picture of the functioning of this group of sensory endings is that there is a population of cells covering with both tonic and phasic responses the whole range of movement of the femoro-tibial joint. In general, the largest fibres show a phasic response and the smaller fibres pure tonic responses; those of intermediate size give an incompletely adapting discharge at a constant position of the joint but show considerable acceleration during movement in one direction and a silent period during movement in the other. Any given tonic fibre has a continuous range of excitation with a maximum at full flexion or extension; the minimum discharge from the whole group of cells is thus at about the mid-position of the joint (Text-fig. 2*a*).

IDENTIFICATION OF CELL POSITION

With the base of the small leg nerve on the electrodes the whole of the flexor tibialis muscle complex in the femur can be removed without abolishing the tonic response to joint movement; the phasic responses are usually absent after this amount of dissection. By careful section of the mid-line of the femoro-tibial hinge it is possible to cut the nerve branches to the tibia without destroying the femoral cells. Such a preparation shows fewer active endings but these include both extensor and flexor fibres. It appears, therefore, that there is no correlation between the location of the smaller cells and the sense of the tonic responses.

External probing with a blunt needle in the hinge membrane excites many of the smaller endings but never brings in the large phasic fibres. By contrast, internal paring of the membrane and adjacent regions of the femur and tibia with a sharp scalpel abolishes all tonic responses but leaves some of the large fibres responsive to rapid joint movement. These results may be correlated with Stuart's observation that the largest cells at the femoro-tibial joint are located far from the membrane surface at the junction of the joint branch with the small leg nerve. At this point the nerve is also joined to a peculiar strand of connective tissue (*s.*, Text-fig. 1) which in dissections resembles a nerve but on histological examination is found not to contain nerve fibres; neither is it a blood vessel, for in arterially injected specimens the small nerve is seen to be supplied by its own small artery running along it from the trochanter and only there joining the main leg artery surrounding the large leg nerve (Milne Edwards, 1872). This connective tissue strand runs from a point on the outer surface of the femur some way from the hinge to the main leg nerve in the tibia where it joins the connective tissue surrounding this nerve-arterial trunk. It seems likely, as suggested by Stuart (1953), that the large cells situated remote from the joint membrane are responsible for the large phasic impulses excited by rapid movement and the small cells near the membrane for the tonic discharges. This correlation, taken with the anatomical picture, suggests further that the large cells are

excited not by any movement or forces at the joint itself but by the internal tensions set up by the movement in the connective tissue. The strand mentioned above is so placed that its length must change during flexion and extension of the joint, whereas the small nerve itself is always rather accurately located between points at the femoro-tibial and trochantero-femoral joints which do not move relative to one another when the segments flex and extend. Text-fig. 1 also shows, at the trochantero-femoral joint, a small, fine muscle with close striation which runs from trochanter to femur and is attached by connective tissue to the small nerve where this runs round the inner curve of the trochanter. It is hard to see that this muscle can have any function other than to hold the nerve in this region away from the cuticle of the trochanter at or very near to the position of zero stretch when this segment moves. Such an arrangement is well suited to provide a fixed reference line for sensory structures responding to movement through the pull of other connective tissue strands whose outer insertion is on moving parts. Possibly some degree of plasticity in the connective tissue is responsible for the phasic nature of the response of these internal endings.

In the scorpion and in *Phrynichus* Pringle (1955) noted that the phasic impulses in large fibres, which were recorded in nerves at the base of the leg under conditions of movement very similar to those here described for *Limulus*, appeared to arise from structures lying deeper in the leg than the lyriform organs responsible for the tonic discharges. Histological investigations were not made in the course of that research, but the course of the small nerve fibre is the same and a similar arrangement of cells may be present in these Arachnids.

THE ADEQUATE STIMULUS FOR THE TONIC RESPONSES

Stuart (1953) showed that the peripheral processes of the small sense cells 'ramify in or between the cells of the hypodermal layer, close to the cuticle'. Bearing in mind the close similarity between the response of these endings and that of the Arachnid lyriform organs the question naturally arises whether the adequate stimulus for the tonic responses is strain in the cuticle near the joint or tension produced in the internal tissues as has been deduced for the phasic responses. Barber (1954), in his study of the proprioceptive endings at the coxo-trochanteral joint, states that no sensilla were seen in the membrane itself, but that the hypodermal cells under which the sense cells lie are less pigmented than those on either side and that the area can therefore be seen through the cuticle from the outside. There is no external sign of sensilla at the femoro-tibial joint, but the possibility is not excluded that some of the finer peripheral processes of the sense cells may penetrate the deeper layers of the cuticle and there react to strains.

Several experiments were performed on the femoro-tibial joint to test this hypothesis, and the results may be summarized as follows:

(1) Recording from the base of the small nerve with the leg intact, the position of minimum discharge in tonic fibres is always with the joint more than half extended; that is, flexor fibres are more in evidence than extensor fibres.

(2) Cutting the distal insertions of the flexor tibialis muscle complex on the inner side of the joint without other damage to the femur shifts the minimum further over towards complete extension, so that little or no discharge in extensor fibres is obtained on full extension.

(3) Cutting off the tibia also shifts the minimum markedly towards extension.

(4) Opening the femur by removal of the lateral cuticle so as to expose the small leg nerve shifts the position of minimum discharge towards flexion, and extensor fibres are now readily excited.

(5) The femoro-tibial hinge is a very free joint and if the flexor muscle insertions are cut there is little elastic bias. Nevertheless, such elasticity as exists is altered by damage to the integrity of either femur or tibia. Thus, with the segments intact there is a slight elastic force tending to produce flexion and this is increased by damage to the tibia; on the other hand, preparations with the tibia intact and the femur opened have a slight extensor elasticity.

(6) In an intact preparation showing a considerable tonic discharge on full flexion, pressure with a blunt point on the outside of the hinge inhibits the discharge and if sufficiently strong brings in extensor fibres.

(7) An isolated joint preparation with femur and tibia completely cut through about 1 cm. from the joint leaving only the nerve intact showed a considerable extensor discharge on full extension. Pressure applied to the cuticle of the tibia so as to expand the section parallel to the hinge line now abolished the extensor discharge and the preparation then showed a large discharge on flexion.

(8) It was always clear in these experiments that the flexor and extensor discharges occurred in different nerve fibres.

It is difficult to explain these results except in terms of the changes in stress produced in the cuticle near the joint by alterations of the mechanical conditions. If the cuticle at the hinge in the intact leg is normally under a small stress due to opposing and nearly equal stresses transmitted by the structure of the femur and tibia, and if the peripheral processes of the sense cells are inserted in the cuticle in two ways so as to respond to strains in opposite sense, then it would be expected that damage to the femur or tibia would alter the natural strain and bias the endings positively or negatively, so changing the position for minimum discharge in the manner found.

A difference must here be noted between the tonic responses from the femoro-tibial joint and those described by Barber (1954) from the coxo-trochanteral joint. In the present series of experiments it was always found that any given ending increased its frequency of discharge steadily as extension or flexion (as the case might be) increased from the point at which the ending was first excited, and that motion had a differential effect (Text-fig. 3). Barber reports that at the coxo-trochanteral joint each tonic ending is excited only over a certain range of joint positions and ceases to fire impulses when the joint is in a position either more extended or more flexed than this range; and that motion in either direction accelerates the discharge. It is possible that this difference in behaviour of the endings at

the two joints indicates a real difference in mechanism and function, but without comparative studies in the same animal the difference does not warrant further discussion.

CONCLUSIONS AND DISCUSSION

It is concluded from these experiments, in agreement with Barber (1954), that there are two types of proprioceptive ending at the femoro-tibial joint of the *Limulus* leg, and that these correspond to the two sizes and locations of sensory cells described by Stuart (1953). Large cells situated at the point of branching of the small leg nerve send processes into an internal connective tissue strand which is stretched by extension of the tibia and give rise to rapidly adapting phasic discharges in large diameter nerve fibres in the small leg nerve. Smaller cells, situated near to the mid-line of the hinge under the outside cuticle of the joint, send processes through or between the hypodermal cells into the lower layers of the cuticle where they react to strains and give rise to slowly adapting tonic discharges; these endings are of two types, some being more excited in the flexed position of the joint and some in the extended position, owing to the different type of strain produced in the two positions.

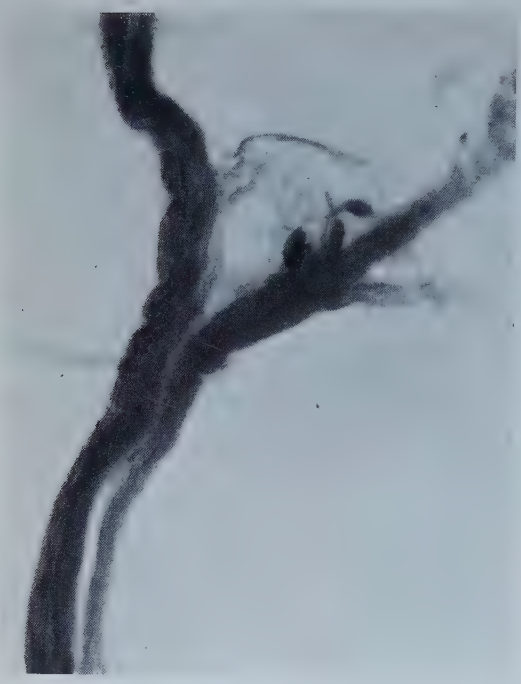
This conclusion points to a homology between the small-cell endings of *Limulus* and the lyriform organs of Arachnids, and suggests that *Limulus* may represent a more primitive stage in the evolution of this type of sensory ending in which cuticular sensilla have not yet arisen by secondary modification of the cuticular products of hypodermal cells. Wigglesworth (1953) has shown that in the campaniform sensilla of insects the sense cell, trichogen cell, tormogen cell and accessory cell arise by differentiation of the grand-daughter cells produced by division of a single hypodermal cell. The origin of the Arachnid sensory cells has not been studied, but if they and the sensilla-forming cells are found similarly to have a common grand-parent the parallel evolution of these two groups of terrestrial Arthropod would be further exemplified. Studies of the histogenesis of the Stuart cells in *Limulus* would then be extremely interesting from an evolutionary point of view.

The large, internally situated, phasic cells cannot be compared directly with anything known in insects unless the chordotonal organs are found to have a similar ontogenesis. The phasic responses described by Pringle (1955) from a scorpion and *Phrynichus* may, however, arise from similarly placed cells. It is possible that the relatively greater reduction of connective tissue in insects as compared with Arachnids has precluded the evolution in the former group of this type of internal proprioceptive sense.

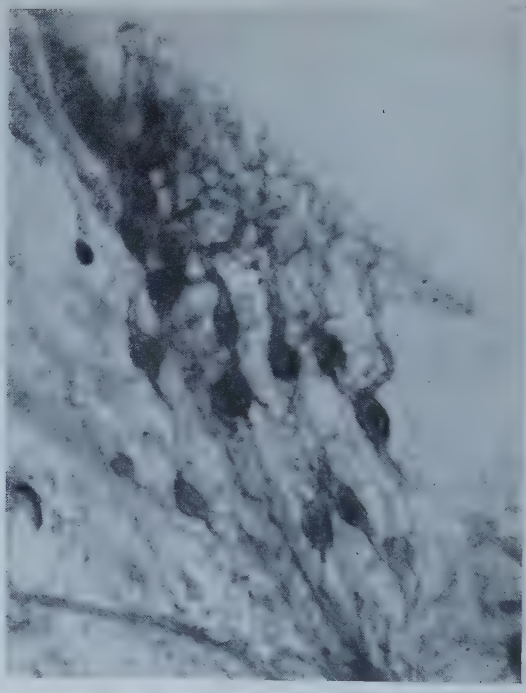
SUMMARY

1. Tonic and phasic discharges of impulses may be recorded in the small leg nerve of *Limulus* from endings sensitive to the position and movement of the femoro-tibial and other joints.

2. The nerve fibres originate from the large sensory cells described by Stuart (1953). Of those at the femoro-tibial joint, one group of cells lies under the hinge



(i)



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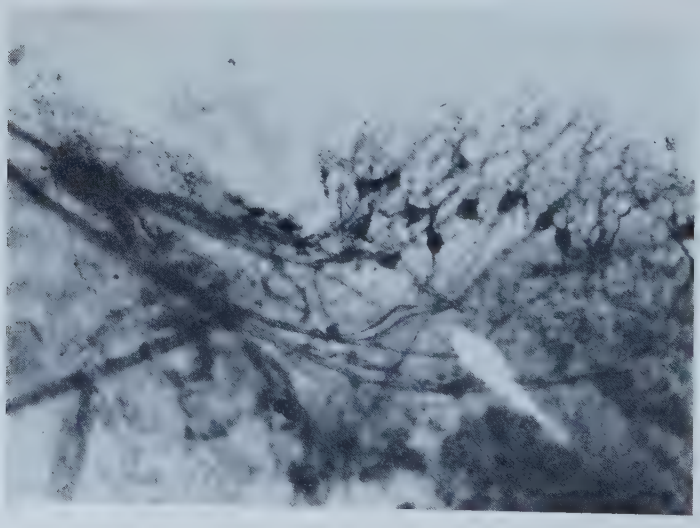

(iii)

PRINGLE—PROPRIOCEPTION IN *LIMULUS*

(*Facing p.* 667)

and the endings react tonically to the strains present in the cuticle; some are excited by flexion and some by extension. The other group of cells, lying more internally, have endings in the connective tissue and react phasically only during joint movement.

3. The relationship is discussed between these endings and the lyriform organs of Arachnids and the campaniform sensilla of insects.

These experiments were conducted at the Marine Biological Laboratory, Woods Hole, during the tenure of Research Associateship of the University of California, Los Angeles. I am very grateful to Prof. T. H. Bullock for allowing me such free use of his apparatus.

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EXPLANATION OF PLATE 9

(i) Group of large neurons located on the nerve branch innervating the femoro-tibial joint. Methylene blue; ×54. (From Stuart, 1953.)

(ii) Proprioceptor neurons from the tibio-tarsal joint; large cell group. Methylene blue; ×90. (From Stuart, 1953.)

(iii) Proprioceptor neurons from the tibio-tarsal joint; small cell group. Methylene blue; ×54. (From Stuart, 1953.)

TETANIC FORCE AND SHORTENING IN LOCUST FLIGHT MUSCLE

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During flight of the desert locust, the dorsal longitudinal depressor of the hindwings produces a prolonged series of non-fusing twitches (Ewer & Ripley, 1953; Buchthal, Weis-Fogh & Rosenfalck, unpublished). This indirect flight muscle is used only for wing oscillations and one may assume that it never contracts tetanically in the animal. The experiments here described were designed to determine the *maximum* alterations in length and force which can be induced in this muscle, i.e. the changes during a tetanus, and do not concern themselves with the question of whether or not the insect makes use of tetanic contractions. The results are used to interpret the changes observed during a twitch (Buchthal, Weis-Fogh & Rosenfalck, unpublished) and are compared with similar figures from frog muscle in order to find out whether the contractile elements differ in the two types of muscle.

For various reasons described in the text it was difficult to produce tetanic contractions of the isolated flight muscle and some unusual precautions had to be taken. The stimulation was indirect through the motor nerve; in fact, direct stimulation of insect muscle does not seem possible (cf. Roeder & Weiant, 1950; Roeder, 1953; Hoyle, 1955). The *potential* force and shortening may therefore be somewhat larger than found here.

1. MATERIAL AND METHODS

The adult desert locusts (*Schistocerca gregaria* Forskål, *phasis transiens* or *gregaria*) were bred at the Anti-Locust Research Centre, London. For reasons given on page 671, it was found essential to keep the locusts at a high day temperature (body temperature 30–40° C.). The morphology of the flight muscle is described elsewhere (Buchthal & Weis-Fogh, 1956).

Saline. See page 672.

Nerve-muscle preparation. The isolated thorax was bisected slightly to the left of the mid-line, leaving the ventral ganglia intact. By means of a wall of adhesive wax (10 g. bees-wax + 4·5 g. natural resin; melting-point *c.* 55° C.), the right undamaged side was firmly fixed to a rectangular slab of plastic (*a* in Fig. 1). The dorsal longitudinal depressor of the hindwing was dissected free, except for its posterior attachment to the third phragma; this phragma is in strong cuticular connexion with the body wall, i.e. with *a*. The anterior, free attachment (second phragma) was fixed to a small clamp and the metanotum as well as the dorsal part of the muscle

were removed, leaving a bundle of parallel fibres of approximately the same length (10% variation). The adjacent structures were cut so that the muscle became free to shorten, but the motor nerve was left intact and still connected to its ganglion in order to prevent neurotrophic degeneration during prolonged experiments. The preparation did not fire spontaneously. Care was taken not to leave the muscle unloaded for any length of time. It was observed that it had to perform a few isotonic contractions against a rather heavy load (20 g.), before constant results were obtained.

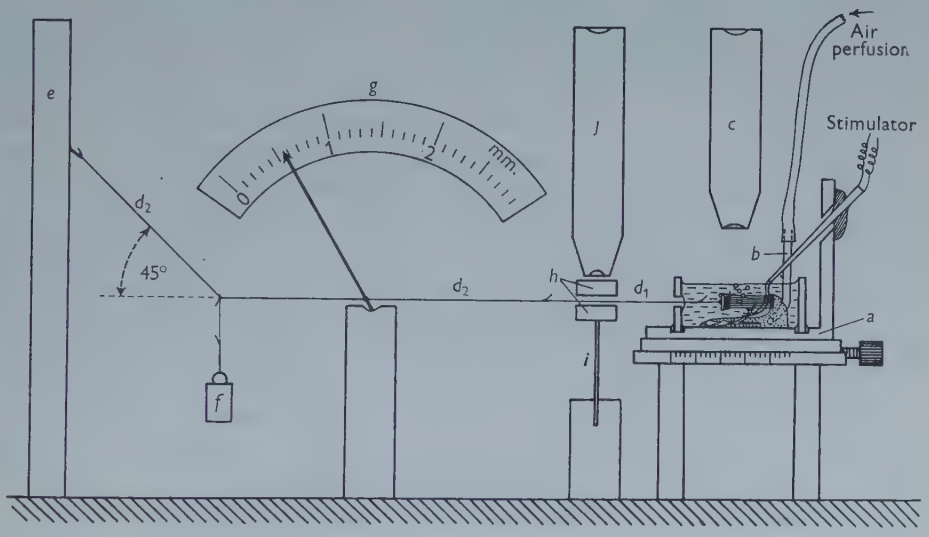

Fig. 1. Arrangement for estimating the isotonic shortening, the isometric extra force, and the resting force at various initial lengths of a flight muscle. Air is blown through the capillary tube (*b*) connected with the third spiracle; it escapes through the cut tracheae at the anterior attachments (bubbles). Both the cantilever *i* and the isotonic lever system *g* were in fact horizontal. For further explanation, see text.

Supply with oxygen. The flight muscle is very sensitive to decreased oxygen tension. During experiments performed at 11° C., the muscle was suspended in air (constant temperature room) and drops of saline were added after each contraction, and sucked away from beneath. In order to prevent collapse of the tracheae due to diffusion of nitrogen, the saline was saturated with atmospheric air instead of with oxygen. At higher temperatures the risk of desiccation made it essential to keep the muscle in saline. The diffusion of oxygen from the surface into the interior was then found to be quite inadequate, and it was necessary to blow air through the tracheal system. The main tracheal supply of the muscle consists of a system of longitudinal tracheae branching off from a large trunk, which originates from the third spiracle; at the anterior end of the muscle these longitudinal tracheae open into a large air sac whose wall is cut during the preparation. The preparation was therefore carried out with the following precaution: the third spiracle, kept open by means of a thin piece of platinum wire (0·08 mm. diameter), was surrounded by

a wall of wax and provided with an extension tube of glass (Fig. 1*b*). The tube projected above the saline and air was blown through the muscle under constant pressure (about 5 cm. of water). It escaped from the cut air sac and the bubbles stirred the saline. The temperature was measured by means of a thermocouple.

Myograph. The myograph is of a simple, static type (Fig. 1); the reading of force and shortening is direct so that the state of the muscle can be followed continuously. The plastic slab *a* with the preparation is placed on the two-way mechanical stage of the microscope *c* which serves to measure the length of the muscle at any applied load (to nearest 0·1 mm.). The muscle clamp is hooked to a straight piece of constantan wire d_1 (0·1 mm. diameter); beyond the second microscope *j* this wire is connected with the thin spun nylon thread d_2. The far end of d_2 is suspended from a movable stage at *e* so that, when a load *f* is applied, the angle between the horizontal part of d_2 and the inclined part is 45°. The muscle is then subjected to a load of *f* grams; by means of the microscope stage, the muscle can be moved several millimetres without any significant alteration of the load. The isotonic shortening is measured by means of a simple lever which moves over the dial *g*; after each change of load, the nylon thread is simply clamped to the lever at a fixed distance from the axis of pivoting. The reading is correct to within 0·05 mm. The isometric force is measured after the constantan wire has been firmly clamped (at *h*) to a damped cantilever *i* of hard steel. The deflexion of the spring is read on an eyepiece micrometer in the microscope *j* (front lens × 44, N.A. 0·8). Strictly speaking, the cantilever system was not isometric, but the external shortening in an 'isometric' contraction *maximally* amounted to 10% of the isotonic shortening measured at the same initial length of the muscle and usually was smaller. When in saline, the muscle was surrounded by a collar with a slit for the wire d_1.

Stimulation. The motor nerve was stimulated from a simple neon tube discharge unit via two platinum electrodes about 2 mm. apart. Neither in respect of the tetanic force nor in respect of the rapid onset of fatigue were better results obtained by 'direct' stimulation (multielectrode) from a square-wave pulse stimulator. The frequency was the lowest which gave a maximum response, i.e. *c.* 30/sec. at 10° C. and *c.* 60/sec. at 25° C. Completely smooth tetani did not occur, but the oscillations were too small to be observed with the present apparatus (cf. Buchthal, Weis-Fogh & Rosenfalck, unpublished). In about half a second the stimulus was gradually increased from zero to well above for maximum response, left on for about half a second to enable a reading to be obtained, and then switched off. Even within this short period, fatigue was found at 11° C., but not at the high temperature. The readings are the maximum changes observed.

Fixation for microscopy. The muscle was prepared as usual, provided with a clamp and suspended vertically in saline at 4° C. and loaded by known weights. The fibres were depolarized by addition of potassium chloride up to 100 mM. K^+ (cf. Hoyle, 1953). The muscle was then killed and fixed by slow, dropwise addition of neutral formalin up to 4% or 5% $CH_3.CHO$. After 24 hr. at 4° C., it was transferred to 7% formaldehyde in saline at room temperature, still with weights attached.

Microscopy. Single fibres were isolated from various parts of the fixed muscle (3–8 days after fixation); this did not alter their length. By means of fine needles each fibre was split longitudinally into six to ten fragments; by comparing the length of the fragments with that of the fibre, it was confirmed that this treatment did not stretch them. Thin fragments, maximally 10 μ thick, usually 2–5 μ, were examined in water under a high-power polarizing microscope (Leitz, crossed Nicols, front lens $\frac{1}{12}$ in. oil immersion). The results were confirmed by measuring single fibrils (sarcostyles, according to Tiegs, 1955) which were always present at the edge of the fragment. The sarcomere height s (= distance between adjacent Z-lines), the height of the anisotropic band A and of the isotropic band I were measured by means of an eyepiece fitted with a screw micrometer (Leitz × 10). In the light from a mercury arc lamp, the accuracy of measurement was about 0·1–0·2 μ.

2. PRECAUTIONS AND SPECIAL PROPERTIES

(*a*) *Survival in saline*

In the preliminary experiments, the adult locusts were kept at a low day temperature of 25° C. during daytime and at 16° C. during the night. They appeared healthy and survived for months, without, however, becoming sexually mature. When different kinds of muscle (abdominal, leg, gut, malphigian tubule) were isolated in a saline similar to that used by Hoyle (1953), they survived well for hours at 25° C. This was not the case with flight muscles, neither large nor small, which had to be kept at 10° C. or below. At 25° C., for instance, the excitability decreased considerably during the first hour and disappeared altogether after 2 or 3 hr. in the saline by which time the isolated muscles were dead. Addition of large quantities of haemolymph had no effect and control experiments showed that the main cause could neither be denervation (cf. Roeder & Weiant, 1950) nor lack of oxygen. The ionic composition of the saline was altered systematically and so was the total osmotic pressure (addition of sucrose), but with negative results. Specific substances like glucose, ascorbic acid, and L-3-oxykynurenin had no effect. Eventually, it was found that addition of crystalline sodium *penicillin* (3 mg./100 ml. saline) made the isolated flight muscles survive for more than 8 hr., even at 32° C.

This unexpected result is not yet satisfactorily explained. It is certain, however, that the effect is not due to penicillin acting directly upon the muscles. It has been known for a long time that locusts of the genus *Schistocerca* are particularly susceptible to infection by the coliform bacterium *Coccobacillus acridiorum* D'Herelle (see Steinhaus, 1946) which often occurs in the alimentary canal. According to Mereshkovsky (1925) and to Pospelov (1926), it is even a normal inhabitant of the haemolymph of locusts kept at 20° C., but Pospelov found that it disappears when the insects are exposed to high temperatures. Accordingly, the cage temperature in my experiments was increased to between 30° and 35° C. (body temperature up to 40° C.). The isolated flight muscles now survived equally well in the aerated saline whether penicillin had been added or not, i.e. for at least 8 hr. at 32° C. It is therefore reasonable to suggest that the muscles were infected *in vivo* and that

the bacteria started to grow at the high temperature when the normal defence mechanisms were removed by isolating the muscle. Proteolytic micro-organisms could then be active at the very surface of the fibres.

The phenomenon is well known in tissue cultures where antibiotics must often be added, but it is also known in the meat preserving industry: in animals which have been subjected to physiological stress before being slaughtered many organs are often infected by various micro-organisms belonging to the normal intestinal flora (Vincent, Veomett & Riley, 1955; Lepovetsky, Weiser & Deatherage, 1953). This raises problems of general interest to students of poikilothermal animals, especially of tropical species like the desert locusts.

The *locust saline* contained 6·5 mM. K^+, 175 mM. Na^+, 2 mM. Ca^{2+}, 2 mM. Mg^{2+}, 161 mM. Cl^-, 10 mM. HPO_4^{2-}, plus 30 mg. crystalline sodium penicillin per litre. The pH was 6·6–6·7 and the saline was saturated with atmospheric air.

(*b*) *Supply of oxygen*

The flight muscle is about 1·2 mm. thick and has a very dense supply of anastomosing tracheae, many of which were cut open so that the inside air was in direct contact with the saline. Nevertheless, the rate of diffusion of oxygen was found to be too small to maintain the normal excitability. In saturated locust saline, the excitability disappeared within 1 hr. at 20° C. and within 15 min. at 30° C. when the muscle was stimulated to give a single twitch at 2 min. intervals. Short tetani (1 sec. duration, spaced by 3 min. intervals) greatly increased the decline, as is seen in Fig. 2: even at 11° C. the muscle must either be suspended in air and drops of air-saturated saline must be applied or the tracheal system of the submerged muscle must be perfused with air. If nitrogen was used for perfusion, the excitability to *indirect* as well as *direct* stimulation disappeared altogether after 10 or 15 min., but the muscle revived in about the same time when air was again admitted. It is not known which part of the neuro-muscular system is the most sensitive to low oxygen tension; probably it is the nerve terminals. The muscle is never subjected to anaerobiosis in the flying animal.

(*c*) *Internal lesions*

When the resting flight muscle is stretched by loads exceeding 40–50 g., it starts to 'give' or 'yield' (Buchthal & Weis-Fogh, 1956). At 11° C., the muscle develops an extra force of about 30–35 g. during isometric tetanus. The critical force for 'yielding' is therefore reached when the stretch corresponds to 10 g. only. It was found that when the total force during activity (= isometric force plus resting force) approached about 45 g., the subsequent isometric force and the isotonic shortening had decreased while the resting length had increased. This 'yielding' is therefore caused by the high tension during tetanic activity and it becomes very marked when the temperature is increased and thereby the tetanic force. At 25° C. as compared with 11° C. the force is doubled or trebled (see page 678), and this specialized muscle is injured by a *single* isometric tetanus at its normal length in the locust and

at the normal body temperature. The isometric force could therefore only be estimated within a limited range of stretch and temperature.

The simplest explanation is that some of the very small fibrils which connect the contractile interior of the fibre with the cuticle, the *tonofibrillae*, break and permit the attached myofibrils to go into the irreversible 'delta state' (Ramsey & Street, 1940). The preparation was found to contract 'spontaneously' only when (1) the attachments were slightly damaged by means of a fine needle, or (2) the muscle had been allowed to shorten so much against a small load that the 'delta state' was induced, or when (3) the total force during activity had reached the 'yielding' force of the passively stretched muscle. The 'spontaneous' firing was therefore correlated with injuries which may lead to excessive shortening of some fibres.

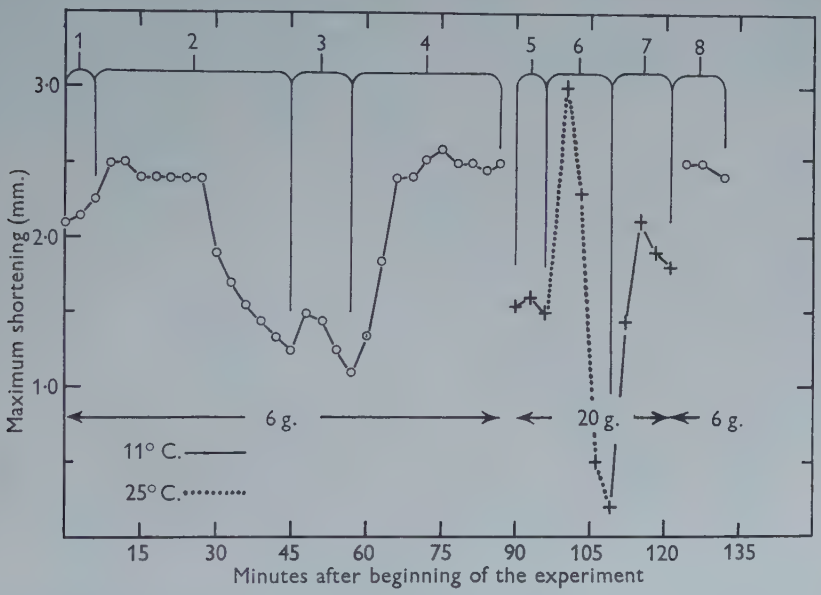

Fig. 2. Each 3 min. the flight muscle performed a short tetanus (1 sec.). The temperature, the load, and the access to oxygen were varied as follows. 1: muscle in air at 10° C., dripping with saline; 2: muscle in saline saturated with air; 3: air-saturated saline is renewed; 4: muscle again in air as in (1); 5: same as (4) but the load is increased from 6 to 20 g.; 6: muscle in air-saturated saline at 25° C. (constantly renewed); 7: muscle again in air at 11° C. as in (1) but load 20 g.; 8: same as (7) but load reduced to 6 g.

(*d*) *Fatigue*

Even in preparations which were perfused with air so that they got sufficient oxygen, the maximum force and shortening always started to decline shortly after the onset of stimulation. At 11° C. this happened within the first second and the decline was rapid, but at 25° C., when the metabolic rate and the work are much higher, the fatigue did not develop until after 5 sec. and the decline was considerably slower. The fatigue is therefore not caused by lack of oxygen.

3. TETANUS AT 11° C.

(*a*) *Example*

Fig. 3 shows a typical experiment; the numbers indicate the sequence of the contractions, each point being the average of three measurements taken at 1 min. intervals at a given length. The deviation between the three readings did not exceed 10%. The first two series of contractions (nos. 1 and 2) fell outside the main course; it was not until the muscle was stretched by a constant load of 30 g. and allowed to

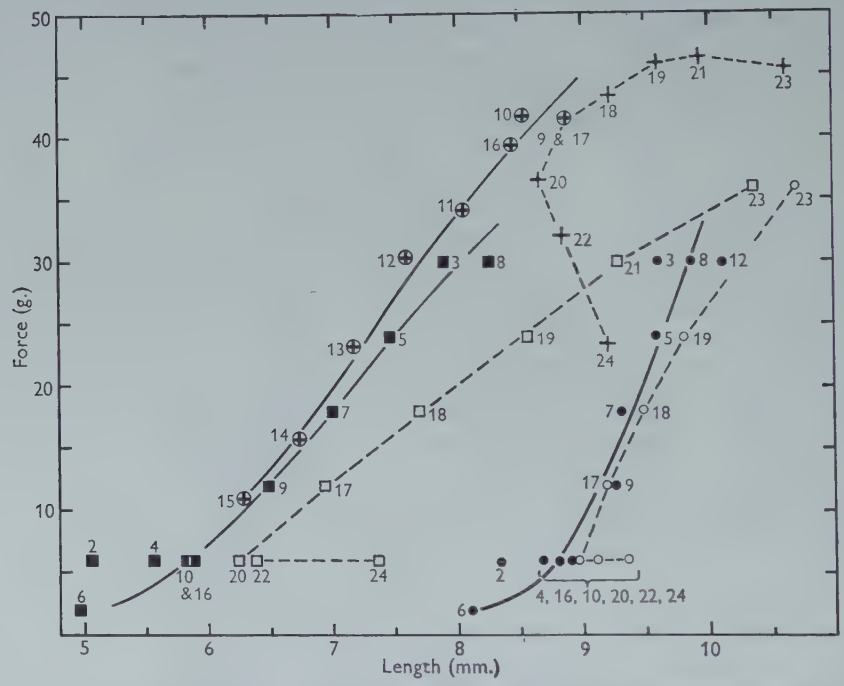

Fig. 3. Typical experiment at 11° C. (tetanus in air, dripping with air-saturated saline). At each length, the muscle performed three isotonic shortenings, the resting length being estimated in between, followed by two or three isometric contractions. Solid curves and filled symbols: before 'yielding'. Broken curves and open symbols: after 'yielding'. The numbers indicate the sequence of contractions. Abscissa: length of longest fibres in preparation (mm.). Ordinate: force due to passive stretch (circles), load during isotonic tetanic shortening (squares), and total force during isometric tetanus (crosses). In grams (g.).

shorten against this load (no. 3) that a reversible course was observed (nos. 4–17; solid curve). When the total isometric force (crosses) had exceeded 42 g., lasting changes occurred (nos. 18–24).

Isometric and isotonic force compared. It is seen that the isometric force (crosses) is higher than the isotonic (squares) when referred to the same length. This is also true after 'yielding' had occurred (cf. no. 23). It must be stressed that the isometric contractions at lengths shorter than the equilibrium length were obtained by moving the preparation so near to the isometric lever that the connecting wire was slack (d_1 in Fig. 1). Upon stimulation, therefore, the muscle shortened against zero

load before it started to develop tension. But, also in this region, the isometric values are higher than the isotonic ones. After the contraction the muscle invariably returned to its former equilibrium length and a plastic 'delta state' was not observed at low temperature. The length-force relationship of flight muscle therefore depends both on the length and on the type of contraction: in conformity with frog muscle fibres, the isometric curve is higher up than the corresponding isotonic curve; this has been interpreted as a type of elastic locking of the contractile system (cf. Buchthal, 1942).

(*b*) *Average length-force diagram*

The average results from eight muscles are seen in Fig. 4. The length (abscissa) is given as a fraction of the length in the body L_b, while the force (ordinate) is in grams. The relationship between force and *passive stretch* (circles) has a steep slope which is nearly constant at lengths above 1·0; the equilibrium length L_0, i.e. the length when no external force is applied, is $0{\cdot}9L_b$. The standard deviation varied from 1·2 to 2·3% of L_b; S.E. averaged 0·8%.

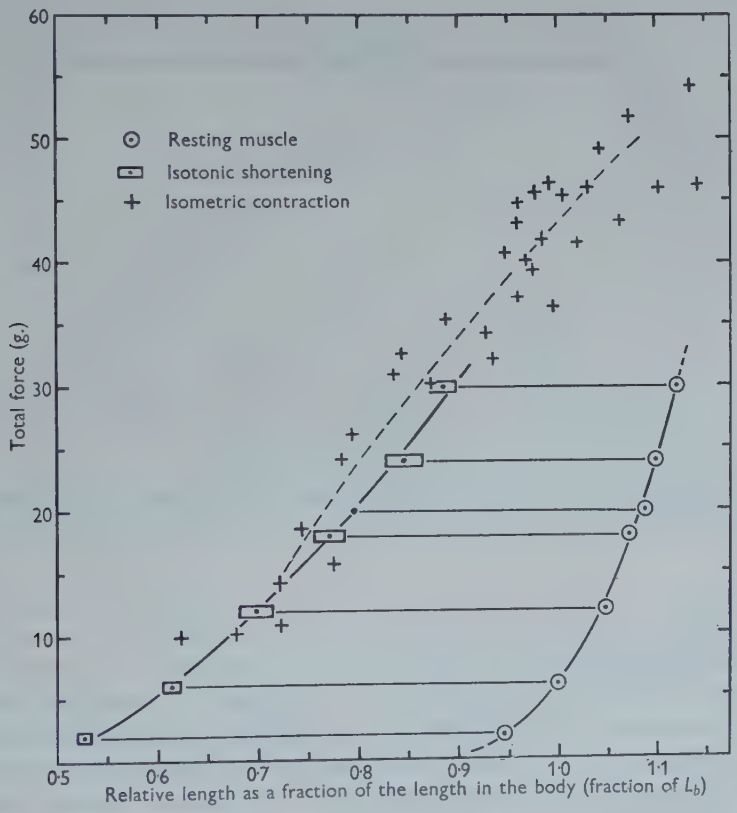

Fig. 4. Average tetanic length-force diagram of eight flight muscles suspended in air at 11° C. The isotonic shortenings are shown by the horizontal lines connecting the initial lengths (circles, radius = S.E.) with the shortest lengths observed (rectangles, length = ½ S.E.). The crosses show the isometric values, each symbol being the average of two or three readings.

Shortening. In normal flight (Weis-Fogh, 1956) as well as in *twitch* experiments on the isolated flight muscle (Buchthal, Weis-Fogh & Rosenfalck, unpublished), it is characteristic that the shortening is very small compared with that of frog muscle, generally about $0{\cdot}05L_b$. Nevertheless, the *tetanic* shortening approached that observed in frog muscle where it amounts to $0{\cdot}7L_b$ before lasting deformations occur ('delta state'). In Fig. 4 the shortenings are shown by the horizontal lines which connect the resting lengths (circles) with the corresponding tetanic

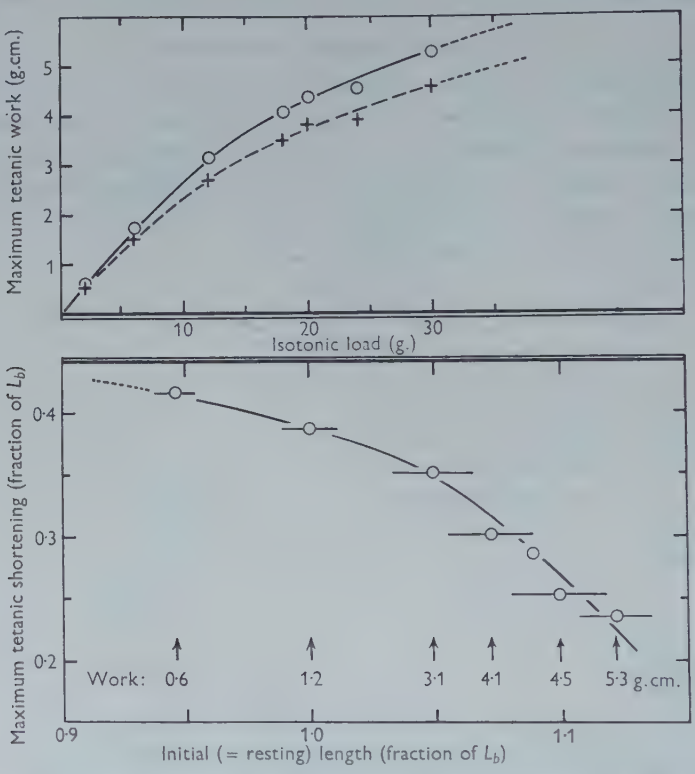

Fig. 5. Average maximum shortening and work during isotonic tetanus of eight isolated flight muscles. The broken curve (crosses) in the upper graph is the work after subtraction of the possible maximum work done by the passive elastic elements. The 'active' work is by far the largest of the two.

lengths (squares; S.E. $0{\cdot}01$–$0{\cdot}02L_b$). The shortening is largest at the smallest loads applied and amounts to $0{\cdot}40$–$0{\cdot}45L_b$.

The crosses connected by the broken curve represent the isometric readings and are further explained in Fig. 6, page 677. Generally speaking, the three curves follow a more parallel course than is the case in frog muscle (Buchthal, Kaiser & Rosenfalck, 1951); unfortunately, the nature of the muscle makes a comparison impossible at high degrees of stretch.

Shortening and work. The isotonic shortening decreases gradually as the length of the muscle increases over the entire range of loads (lower part of Fig. 5). But the

relation is such that the total mechanical work which the muscle is capable of doing (= load × shortening) increases from zero at equilibrium length to about 5 g.cm. at the highest loads applied (solid curve in upper part of Fig. 5). An extrapolation shows that maximum work is hardly reached within the *biological* range of load or stretch, i.e. below the 'yielding' length: the higher the load the more work can be done. In frog muscle a maximum is present (Buchthal *et al.* 1951) but the locust muscle is damaged before the stretch reaches such an extent.

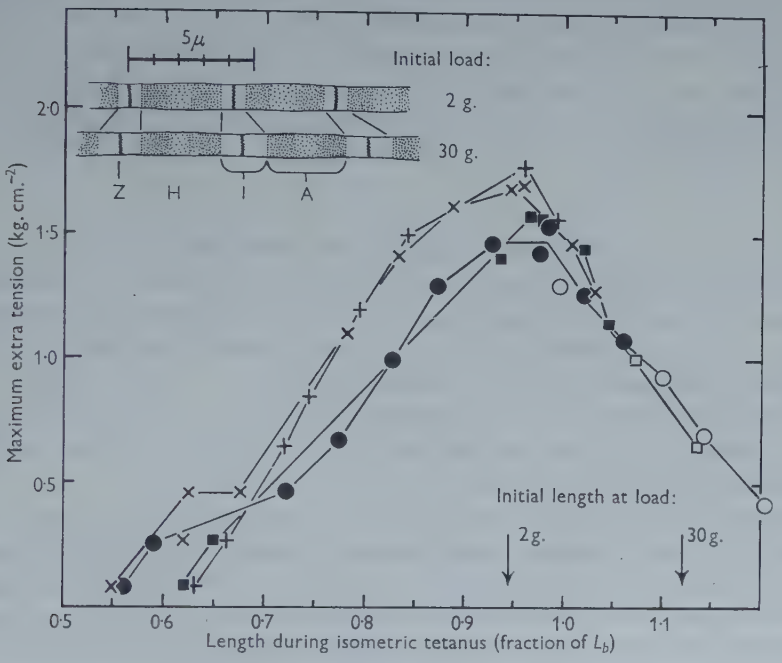

Fig. 6. Maximum increase in tension during isometric tetani at 11° C. in four isolated flight muscles (cf. the four symbols). The tension is calculated as F/S_b, where F = extra force and S_b = cross sectional area at L_b (see Buchthal & Weis-Fogh, 1956). The microscopical structure of two isolated myofibrils is sketched in the left corner. The upper fibrii derives from a muscle which was stretched by 2 g. and the lower from one stretched by 30 g. prior to and during fixation. The upper fibril is near to the relative length at which maximum extra tension is developed ($0{\cdot}96L_b$).

The broken curve in Fig. 5 is the total external work done by the muscle after the work for passive stretching has been subtracted. The true work done by the contractile system must be somewhere in between, depending on the speed of shortening and on the time allowed for adjustment. It is of course not permissible to use the isometric force-length relationship for calculating the work (cf. Ramsey & Street, 1940) because of the difference between the isotonic and the isometric curves seen in Figs. 3 and 4. The maximum work of a tetanically contracting frog muscle fibre is about $0{\cdot}6P_0L_0$, where P_0 approximates to the maximum isometric force (extra force) and L_0 is the equilibrium length (Buchthal *et al.* 1951); it occurs at a load of *c.* $0{\cdot}6P_0$. At 11° C. the maximum work which has been obtained from a flight

muscle is only $0{\cdot}22P_0L_0$, but this figure is doubled at high temperature (cf. next section).

Isometric force. In Fig. 6, the extra tensions (in kg.cm.$^{-2}$) developed during isometric tetanus have been plotted against the length of the four muscles used. At the right-hand side of the graph the open points refer to lengths at which 'yielding' had occurred. The true slope of this part of the curve must therefore be assumed to be less steep. The extra tension decreases rapidly at both sides of the maximum zone at $0{\cdot}96L_b$. Thus, the region where the greatest *extra* force can be produced does not coincide with that of maximum *tetanic* work. But if we consider the *total* force (Fig. 4), which is what matters in the animal, both this and the work increase with length. At 11° C. the maximum tension amounts to 1·6 kg.cm.$^{-2}$ which is considerably less than in frog muscle fibres: 2·8 kg.cm.$^{-2}$ at 0° C. and 3·3 kg.cm.$^{-2}$ at 20° C. (Casella, 1951).

4. EFFECT OF INCREASED TEMPERATURE

It has not been possible to compare frog muscle and flight muscle in detail because too little information is available. It is clear, however, that the tetanic force of the flight muscle increases more with increasing temperature than has been found in frog muscle (Buchthal *et al.* 1951; Casella, 1951).

At a given pull the length of a *resting* frog muscle increases by less than 0·5% per 10° C. increase in temperature (Buchthal *et al.* 1951). In the flight muscle the figure was as high as 2–3% per 10° C.

Exps. 5 and 6 in Fig. 2 show that the *isotonic shortening* against a load of 20 g. was doubled when the temperature was increased from 11 to 25° C. The relationship could only be studied at rather high loads, i.e. between 20 and 40 g. The shortening was then found to increase about 1·9 times from 15 to 25° C. and 1·6 times from 25 to 35° C. In other words, the tetanic shortening and the *work* done is nearly tripled when the temperature was increased from 11° C. to the normal body temperature for flight. At smaller loads, another phenomenon hampered the experiments: the excessive shortening caused lasting injuries resembling those which characterize the so-called 'delta state' of frog muscle fibres (Ramsey & Street, 1940). Even with zero load, such excessive shortenings did not occur at 11° C.; at 35° C. the muscle was injured if the load did not exceed 20 g. The critical length was $0{\cdot}40$–$0{\cdot}45L_b$; the maximum permissible shortening in this muscle is therefore $0{\cdot}55L_b$.

During an *isometric* tetanus, the *force* could not be estimated directly at temperatures higher than about 20° C. because the 'yielding' force was then exceeded. If, on the other hand, we use the isotonic experiments for estimating the pull which the muscle could exert after having shortened to a given length, the force was doubled at 25° C. and tripled at 35° C. compared with 11° C. Since, as in frog muscle, the isometric force is somewhat larger than the isotonic force (cf. Figs. 3 and 4), this means that the *isometric tensions* would not be less than 3 kg.cm.$^{-2}$ at 25° C. and 4 kg.cm.$^{-2}$ at 35° C., if one had been able to measure them. This is of the same

order of magnitude as in skeletal muscle of vertebrates. In mammalian muscle, moreover, the increase in force with temperature is as pronounced as in locust flight muscle; it seems to be a specific property of the structural proteins in question, since both the force and the temperature dependency are retained after the enzymes have been removed by glycerol extraction and the 'model' is activated by means of adenosine triphosphate (cf. Weber, 1955).

There is therefore no apparent difference in the potential force and work of locust flight muscle as compared with ordinary skeletal muscle.

5. STRUCTURE

In the fixed flight muscle the myofibrils were about 1 μ in diameter. In *thin* (2–8 μ) fragments of fibres, the A-bands, the I-bands and the Z-lines were all distinct under the polarizing microscope ($\frac{1}{16}\lambda$ compensator). With increasing thickness, the transition became less well defined. The H-zone, on the other hand, could not be distinguished sufficiently well to make measurements profitable; it could easily be seen as a less dark part in the middle of the A-band, occupying about one-third of the latter. These structures have recently been identified in flight muscles of locusts and cockroaches by means of the electron microscope (Edwards, Santos, Santos & Sawaya, 1954*a*, *b*). The drawings in Fig. 6 show the appearance of two fibrils; the upper fibril is near to its equilibrium length while the lower one is stretched.

Length of A- and I-bands. Muscles were fixed at different degrees of passive stretch and fragmented in a Waring blendor. The results support those given below, but, because of the uncontrolled treatment in the blendor, they were considered less reliable than the results seen in Fig. 7; the latter derived from five females of the same size and taken from the same batch. The five muscles were stretched by 2, 6, 12, 15 and 30 g. respectively during the entire period of fixation (48 hr.), and six to ten fibres were isolated from various parts of each muscle. The average sarcomere lengths (= distance between two neighbouring Z-lines) and the average lengths of the A- and I-bands were measured on different fragments from each fibre (not less than ten estimates). Each point in Fig. 7 represents an average value from one fibre. The number of circles indicates the number of fibres found to have the same average. It is seen that the I-band of the passive muscle increases in length from 0·9 μ at 2 g. load to 1·7 μ at 30 g. load, the intermediate loads giving values in between. The A-band, on the other hand, remains constant and does not become stretched. When the load on a resting flight muscle is increased from 2 to 30 g. its length increases by about $0{\cdot}17L_b$ (Fig. 4). According to Fig. 7, the same increase in load stretched the I-bands by 0·8 μ or by 20% of the sarcomere length in the body, without affecting the A-bands. The increased length can therefore be accounted for by an increase of the I-bands alone. This finding is in accordance with recent results by A. F. Huxley & Niedergerke (1954) and by H. E. Huxley & Hanson (1954). They are in contradiction to the conclusions of Buchthal, Knappeis & Lindhard (1936) and of Carlsen & Knappeis (1955). It is not known how the microstructure

of the flight muscle is altered during activity but the many similarities between vertebrate and insect muscle make it most likely that the changes are similar to those described by A. F. Huxley & Niedergerke (1954) and H. E. Huxley & Hanson (1954). According to them, the A-bands remain of constant length until the I-bands have disappeared. Fig. 7 is supposed to show the distribution of the I- and A-substance when a flight muscle *starts* to contract isometrically. At the length in the body, the average sarcomere measured $s = 4{\cdot}05\ \mu$. Maximum of isometric force occurred at $I/s = 0{\cdot}21$; i.e. the ratio is smaller than found in frog muscle (cf. Table 1). It must be stressed that the shortening in a twitch, i.e. during natural activity, is so small that the ratio will not go much below this value.

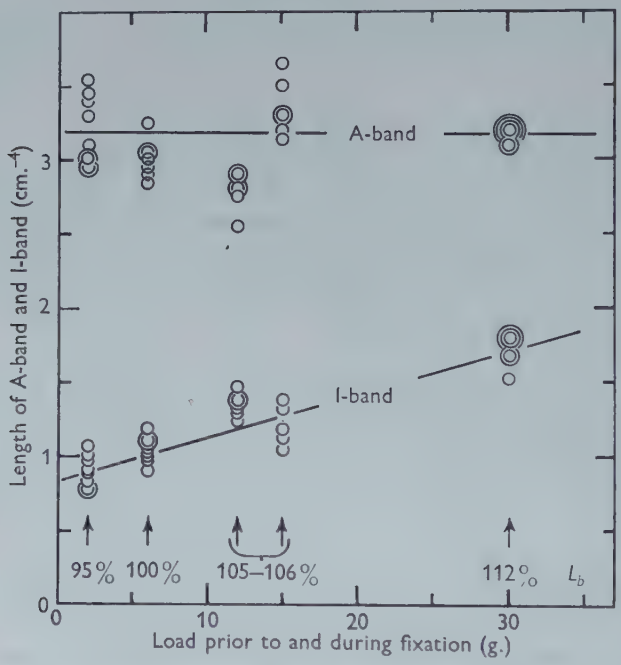

Fig. 7. The length of the optically isotropic (I) and the anisotropic (A) bands in five flight muscles. Within the degree of accuracy, the increase in length can be accounted for by the increased length of the I-bands alone.

6. DISCUSSION

The flight muscle does not contract tetanically in the locust and it shortens very little (about 5%) during natural twitch contractions. It is therefore surprising to find that its contractile substance is able to shorten to nearly the same extent as that of vertebrate muscle and it can also produce as high tetanic tensions. There are some differences, however: (1) The sarcolemma has less mechanical strength in insect than in frog muscle fibres (Buchthal & Weis-Fogh, 1956). This seems to account for the linear (non-exponential) length-force relationship of resting insect muscle; this relationship need therefore not imply that the contractile interior differs in the two muscle types. (2) The extensibility of resting flight muscle of

insects is considerably smaller than that of frog muscle (Buchthal & Weis-Fogh, 1956). But in insects, the flight muscles cannot be stretched much above their natural length because of the strong cuticular box in which they are suspended. (3) In the flight muscle, the greatest tetanic force is reached at the length in the body, as in frog muscle, but at a lower ratio between the length of the I-band and the length of the whole sarcomere (I/*s* ratio).

One may add that insect muscle fibres only seem to be excitable via the nerve terminals. However important this is for the understanding of their function it should not conceal the fundamental similarity found between the mechanical properties of the *contractile* systems in the two types of muscle. The mechanical changes during a twitch of the flight muscle differ in many ways from those seen in frog muscle and will be described elsewhere. In the light of the present results most of these differences can be attributed to differences in passive-elastic and recovery mechanisms rather than to any fundamental difference in the contractile system.

The structure of the flight muscle fixed at various lengths showed the same changes with passive stretch as has recently been observed in frog muscle (A. F. Huxley & Niedergerke (1954), interference microscopy of living fibres; H. E. Huxley & Hanson (1954) and Hanson & H. E. Huxley (1955), microscopic observations on isolated fibrils treated in a variety of ways). An outline of their theory on the correlation between structure and function of striated muscle is as follows: an A-band consists of an array of longitudinally arranged rodlets (myosin); their length remains unaltered both during passive stretch and during activity, provided the shortening does not reach such an extent ($0{\cdot}65L_b$) that the I-bands have disappeared and contraction bands begin to develop. The I-band is subdivided by the Z-disk and consists of longitudinal filaments (actin) which extend into the A-band up to the H-zone where they are attached to a system of passive elastic filaments (S-filaments). The main point is that, during activity, the I-filaments are drawn into the A-bands between the rodlets. The isometric extra force in a tetanus depends on the number of contact points between the I-filaments and the A-rodlets. In this simple model the isometric force is determined by the length of the overlap between actin and myosin filaments in each sarcomere. If the muscle is allowed to shorten, a point is reached when the whole length of the I-filaments is drawn into the A-bands, the I-bands 'disappear', and contraction bands begin to form; further shortening would then imply a curling up of the elements.

According to this model one should expect a linear decrease of the extra force when the muscle is stretched above and a more complex decrease when it is allowed to shorten below the length of maximum force. The above type of variation of the extra force was observed by Ramsey & Street (1940); the general tendency is the same in locust flight muscle (Fig. 6), but the relationship could not be followed to the extreme degrees of stretch. Table 1 shows how the structural data are correlated with the mechanical findings in frog muscle fibres and in the flight muscle. In the latter case it has been assumed that the length of the A-bands remains constant during an isometric tetanus. The values for maximum tetanic force are printed in

bold type. It is seen that maximum of force is reached at a lower l/s ratio in locust flight muscle (0·21) than in frog muscle (0·40); when the I-bands disappear ($l/s = 0$) the force is much reduced but still appreciable. The flight muscle is not capable of being stretched nearly as much as the frog muscle and extra tension is likely to disappear at about $1{\cdot}25L_b$.

Table 1. *Comparison between frog muscle fibres and the flight muscle of the desert locust*

Muscle	Length as a fraction of		Isometric tension (kg.cm.$^{-2}$)	Length (cm.$^{-4}$) of			l/s
	L_0	L_b		Sarcomere (s)	A-band	I-band	
Fibre of frog	0·65	0·43	0·5*	1·5†	1·5†	0 †	0
	1·00	0·67	2·3*	2·0‡	1·5‡	0·5‡	0·20
	1·25	**0·83**	**2·4***	**2·5‡**	**1·5‡**	**1·0‡**	**0·40**
	2·10	1·40	0 *	4·2‡	1·5‡	2·7‡	1·08
Flight muscle (11° C.)	0·85	0·77	0·8	[3·1]	[3·1]	[0]	[0]
	1·00	0·90	1·5	3·6	3·1	0·5	0·13
	1·07	**0·96**	**1·6**	**3·9**	**3·1**	**0·8**	**0·21**
	1·17	1·05	1·1	4·1	3·1	1·3	0·33
	1·25	1·12	*c.* 1 (?)	4·9	3·1	1·7	0·44

L_0 = equilibrium length = length when no load is applied. L_b = length in the body (in frog, cf. Hill, 1949). Bold type: length where maximum extra force is developed (Ramsey & Street, 1940). Brackets: values extrapolated from Fig. 6.

* Casella's force measurements (1951; 0° C.) combined with length-force diagram in Buchthal *et al.* (1951).

† H. E. Huxley & Hanson (1954).

‡ A. F. Huxley & Niedergerke (1954).

As to the qualitative correlation between structure and function the present findings are in good agreement with the above model, but there are two quantitative differences which need comment, namely the decreased extensibility and the low l/s ratio of the flight muscle. The small extensibility of insect flight muscle is without much significance as long as the possibility exists that it is determined by the strength of elastic components parallel to the contractile elements or by the strength of the attachments (tonofibrillae). If it *is* caused by elastic elements in series with the contractile elements, it is most reasonable to identify them with the S-filaments of the H-zone. This elastic element is supposed to be passive and should not be confused with the series elastic element of Hill (1949). As to the extra force, it should depend on the distance of overlap between the actin and the myosin filaments and not directly on the l/s ratio. At a given ratio the overlap is determined by the length of the S-filaments. Unfortunately too little is known about the H-zone in insect flight muscle to justify further speculation, but it is tempting to suggest that some of the mechanical differences between frog muscle and locust flight muscle are caused by the different properties and proportions of the non-contractile material rather than by differences in the contractile elements themselves.

7. SUMMARY

1. The maximum tetanic force and shortening was studied in the isolated, dorsal-longitudinal muscle of the hindwings of the desert locust (*Schistocerca gregaria*).

2. The muscle tended to die when isolated in artificial salines but after penicillin had been added it survived well for more than 8 hr. at 32° C. The cause is discussed.

3. The flight muscle is very sensitive to lack of oxygen and, at room temperature and above, it is necessary to perfuse the main tracheae with air. No difference was observed between indirect and direct stimulation.

4. The natural activity consists of repetitive twitches. Tetanic contractions always caused rapid fatigue and tended to injure the muscle, partly because of excessive shortening ('delta state') and partly because the muscle 'gave' at the comparatively small total tension of 2–2·5 kg.cm.$^{-2}$.

5. The length-force diagram at 11° C. resembles that of ordinary skeletal muscle, but the maximum shortening ($0{\cdot}45L_b$) and the maximum isometric tension (1·6 kg.cm.$^{-2}$) are smaller than in frog muscle at that temperature. As in mammalian muscle, the isometric tension increases much with temperature and was estimated indirectly as 3 kg.cm.$^{-2}$ at 25° C. and 4 kg.cm.$^{-2}$ at 35° C. The largest observed shortening was $0{\cdot}55L_b$. At ordinary body temperature, tension and work are of the same magnitude as in skeletal muscle of vertebrates.

6. The flight muscle is less extensible than frog muscle and the maximum isometric force is produced at a lower I-band/sarcomere ratio (0·2).

7. As in frog muscle, passive stretch only affects the I-bands, while the A-bands remain at constant length, at least in the fixed material used here.

8. It is probable that the mechanical specialization of the flight muscle mainly concerns the sarcolemma and the other passive-elastic elements; the contractile elements do not seem to differ from ordinary muscle.

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ENDOCRINE ACTIVITY DURING INSECT EMBRYOGENESIS. CONTROL OF EVENTS IN DEVELOPMENT FOLLOWING THE EMBRYONIC MOULT (*LOCUSTA MIGRATORIA* AND *LOCUSTANA PARDALINA*, ORTHOPTERA)

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(With Plate 10)

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INTRODUCTION

In a previous paper on the problem of endocrine activity during insect embryogenesis (Jones, 1956) an endocrine system, composed of the brain and a pair of ventral head glands, was described for locust embryos and shown to be responsible for inducing the moult.

In the case of the embryos of *Locustana pardalina* and *Locusta migratoria* the ventral head glands were formed and became functional when growth by cell division was already three parts completed. This stage was reached after the eggs had been developing for 8–9 days at 30° C. The embryo had already completed the process of revolving around the posterior pole, namely ketatrepsis, and had reached a length equal to three-quarters that of the egg.

When the embryo had reached a length about equal to that of the egg, the moult had already taken place. Growth by cell division ended 3 days later, that is to say, on the 12th day of the 14–15 days required for the embryo to reach the stage when it was ready to emerge (Table 1).

In the case of the diapause eggs of *pardalina*, the renewed burst of mitosis at the end of diapause was initiated before the ventral head glands were formed. It was thus evident that these glands were not responsible for terminating diapause, nor with setting in motion the process of development at this stage. The findings led to the suggestion that these glands in the locust embryo appeared to be exclusively concerned with the moult (Jones, 1956).

It is of interest to recall that Seidel (1936) demonstrated the presence of a differentiation centre in the early insect embryo. The centre is essentially physiological in nature, and according to experimental evidence it has its focal point in the anterior part of the presumptive prothorax. Moreover, it had been assumed up till now that just such a centre as this controlled growth and differentiation throughout embryonic development.

However, in view of the fact that an organized neuroglandular system is responsible

for the moult in locust embryos (Jones, 1953) there remained the possibility that development during the post-moult period depended upon the secretory activity of this same hormonal system. Indeed, this possibility was strengthened by the findings of Williams (1952) on the silkworm *Cecropia* in his studies on the endocrine control of metamorphosis. He provided substantial proof that metamorphosis is set in motion by the active principle released by the thoracic glands (ventral head glands, lateral cells of Weismann's gland) under the tropic stimulation of a brain factor.

The influence of the ventral head glands in locust embryos upon events during late embryogenesis has therefore been examined in more detail in the present work. Attention has also been focused on the possibility that during late embryogenesis these same glands may control the activity of the pleuropodial glands and the liberation of the mechanism responsible for the deposition of melanin pigments.

METHOD

The present investigation is based on ligaturing experiments performed on *in situ* locust embryos of *Locusta migratoria* and *Locustana pardalina*.

Eggs chosen for study were those in which the embryo had completed its revolution and had attained a length about two-thirds that of the egg (Table 1). The reason for this choice of material was that previous ligaturing experiments showed that the 'critical period' for the moult occurred soon after this stage in the growth of a locust embryo (Jones, 1956).

In the case of *migratoria*, the eggs were obtained from fertile females kept in cages. The embryo in these eggs reached the stage mentioned above about 7–8 days after oviposition when kept moist at 30° C. On the other hand, in the case of *pardalina*, dried dormant eggs were taken from stock and these reached the stage required about 5 days after wetting and keeping them at 30° C. (Table 1). However, since locust eggs, even of the same pod, tend to develop at different rates one could not rely absolutely on these times. For this reason, the outer opaque chorion of the eggs was removed a day or so beforehand by immersing them in a saturated bleach solution to expose the transparent serosal cuticle. It was thus possible to examine the growing embryo in the egg and to determine its position and state of development with considerable precision.

Locust eggs when kept moist at 30° C. are highly turgid, and if attempts are made to ligature them in this state, they invariably end in the egg bursting. However, it was noticed that if eggs were kept at 10° C. for about 24 hr. after being kept at the higher temperature the extra-embryonic fluid tended to escape through the serosal cuticle, and in this way, the eggs were rendered less turgid.

The ligaturing technique was consequently applied to eggs kept beforehand at a low temperature. Moreover, when the ligature was gradually tightened the pressure was relieved by a further escape of fluid through the serosal cuticle. Thus it was possible, without bursting the egg, to divide the embryo into blood-tight compartments.

Changes in growth which had taken place in the separated parts of ligatured embryos and in their controls were estimated by observing the embryos *in situ* and by dissection. Details of changes were determined by an examination of serial sections. The principal stains used were Mayer's haemalum and eosin.

EVENTS IN THE EMBRYONIC DEVELOPMENT OF A LOCUST

Table 1 is an approximate time-table of events in embryonic development based partly on the results of the previous study already mentioned, and partly on further observations. It is meant to show, in particular, how the times when the endocrine centres are formed and become active are related to significant events such as moulting, the digestion of serosal cuticle and the deposition of pigments.

Table 1. *Approximate timing of events in the development of diapause eggs of* Locustana pardalina *and of non-diapause eggs of* Locusta migratoria *at* 30° C.

Days after wetting the quiescent egg* of *L. pardalina*	Events of development	Days after oviposition of the egg of *L. migratoria*
1	—	—
2	Egg turgid	—
3	Mitosis begins	—
4	Neurosecretary cells active	—
5	Corpora allata and ventral head glands take shape. Revolution of embryo completed	8
6	Maximal activity of glands. Moult	9
7	Deposition of melanin begins. Activation of pleuropodia	10
8	Mitosis ends	11
9	Digestion of serosal cuticle completed	12
10	—	13
11	Embryo emerges	14

* At the end of diapause the egg remains in a quiescent state if kept dry. On wetting, the egg recommences its development.

THE VENTRAL HEAD GLANDS AS THE SOURCE OF THE HORMONE CONTROLLING LATE EMBRYOGENESIS

In order to prevent the moult taking place in the thorax and abdomen of a locust embryo it is necessary to ligature between the head and thorax before sufficient hormone has been released from the neuroglandular system. If the ligature is applied between the thorax and abdomen, the moult is limited to the head and thorax (Jones, 1956). It was also shown in this previous work that the ventral head glands reached their maximal size and activity at the time of the moult. Thereafter, they became reduced in size (Table 1). However, there was reason to suppose, on the basis of histological evidence, that they were functional during the post-moult period up to the end of mitotic growth in the embryo.

Locust embryos were therefore ligatured *in situ* before and after the critical period for the moult, in order to examine in further detail the effect upon the growth of the separated parts. It transpired from the previous study (Jones, 1956) that the critical period for the moult occurred in embryos when they were about three-quarters the length of the egg; in embryos about two-thirds the length of the egg the critical period had not been reached. Ligaturing experiments on embryos were therefore planned accordingly.

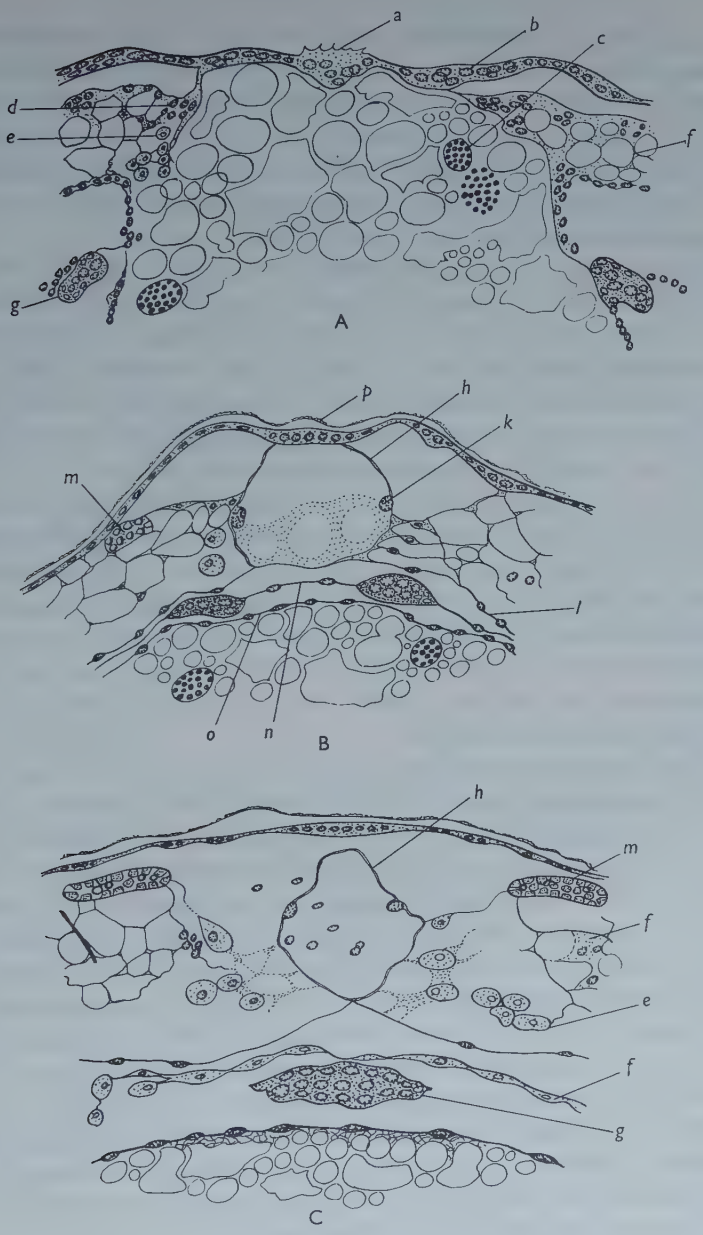
The ligature was applied to the egg so as to separate the embryo at a particular level into two blood-tight compartments. The ligatured eggs were kept at 30° C. and relatively few failed to survive the operation. Indeed, the separated parts of the embryo remained alive for up to 3–4 weeks, well beyond the time required to obtain confirmatory results. The heart continued to beat rhythmically and this served as a valuable indication of the healthiness of the separated parts of the ligatured animal.

Of a total of thirty-four eggs ligatured between the thorax and abdomen before the critical period only six failed to survive in health for up to 8 days following the operation. Of the survivors, the abdomen failed to moult and showed no signs of having progressed further in its growth. On the other hand, the head and thorax both moulted and showed a marked advance in their growth and development, although it was not so great as that in controls over the same period.

In the anterior compartment of the ligatured egg the serosal sac continued to contract as a consequence of which the yolk was pressed into that part of the prospective gut which was present in the thorax. The head and thorax, with the yolk available, progressed more or less normally in their growth and development. Pigmentation of the presumptive eye-disk and the segmentation and pigmentation of the various parts of the head and thorax had in fact advanced in 8 days almost up to the stage normally reached by the embryo a few days before it is ready to emerge (Pl. 10 B_1).

In contrast, the abdomen had not advanced any further in its development than the stage it had reached when it was separated off from the thorax. The appearance of the gut and the size and number of the Malpighian tubules confirmed that development had come to a halt in the abdomen after the ligature had been applied (Pl. 10 B_2). In sections, the remains of the amnion were still present in the mid-dorsal line. Though the dorsal closure was practically completed, the heart was still at the stage of being represented by groups of cells and lateral sinuses on each side of the yolk mass. The gonads were present as a pair of organs, each to one side of the yolk mass (Text-fig. 1 A).

Of a total of twenty-six embryos ligatured between the head and thorax before the critical period, eight were discarded as unsuccessful. Of the remainder, the head in most cases appeared to remain healthy up till the end of the 8 days following the application of the ligature. Though the head did not increase its size, two processes sometimes occurred to indicate an advance in development: increased pigment migration over the eye-disk and an advance in segmentation. On the other hand, the thorax and abdomen failed to moult and internally there was no evidence of progress in growth and development (Pl. 10 A_1, A_2).



Text-fig. 1. Transverse sections through the 4th–5th abdominal segments of embryos of *Locusta migratoria* ligatured between the thorax and abdomen before and after the critical period for the liberation of the development hormone to show in particular the related state of development of the heart and male gonads. A: ligature applied before the critical period. Laterally placed cells and sinuses representing the heart and the gonads lie one on each side of the yolk mass. B: ligature applied just after the critical period. The heart is a single median sinus and the gonads lie separately below and to the side of the sinus. C: ligature applied 3 days after the critical period. The heart possesses its own wall and below it the gonads have fused to form a median organ. *a*, remains of amnion; *b*, epidermis; *c*, yolk-cell nucleus; *d*, cardial cells; *e*, pericardial cells; *f*, fat body; *g*, gonad; *h*, wall of heart; *k*, cardioblast; *l*, pericardial membrane; *m*, muscle; *n*, inter-gonadal membrane; *o*, gut epithelium; *p*, shed cuticle.

Efforts were next made to determine the effect of ligaturing embryos between the thorax and abdomen, after the critical period for the liberation of the moulting hormone. In the case of the head and thorax, there was a marked advance in development, the stage reached corresponding to that normally expected in embryos a few days before they are ready to emerge (Pl. 10 C_1). In the case of the abdomen, progress in development, though more limited, had also taken place. There had been a moult, the heart reached the stage of being a tubular sinus along the mid-dorsal line and the posterior segments were pigmented.

Internally, the midgut was rounded, the hindgut was relatively long and the Malpighian tubules had increased in number and were long and thread-like (Pl. 10 C_2). As illustrated in Text-fig. 1 B, sections of the abdomen showed that the heart had developed into a mid-dorsal sinus, dorsally bounded by the epidermis. The cardioblasts were placed laterally. The pair of gonads lay separately, each below and to one side of the heart sinus and between the pericardial membrane and the gut epithelium. They were joined together by a median membrane.

Though there was a marked advance in the development of the abdomen after it had been separated off from the rest of the body just after the critical period, it was significant that there was no further advance in development in the abdomen, even when the ligatured embryos were kept for periods up to 10–14 days. Accordingly, it was assumed that when the embryo was ligatured between the thorax and abdomen just after the critical period the amount of growth factor which had previously entered the abdomen and become available to the cells was insufficient for development to progress further than it did.

In view of the preceding assumption embryos were first partially ligatured. They remained in this condition for 3 days after the critical period before the ligature was tightened so as to divide the embryo completely between the thorax and abdomen.

In these experiments the abdomen advanced considerably in its development. For instance, the heart could be seen along the mid-dorsal line as a well-formed tubular organ and pigment deposition extended well forwards over the abdominal segments. Internally, the gut and Malpighian tubules reached a stage in their development corresponding to that normally reached in the embryo when it is about to emerge. In sections of the abdomen the heart was shown to have reached the stage of having its own wall, and the gonads were fused to form a median organ which lay between the heart and the gut epithelium (Text-fig. 1 C).

It is apparent, therefore, that the factor released by the neuroglandular system not only initiates growth and development in the embryo, but that its production is needed for about 3 days after the critical period to stimulate progress in development up till the time the embryo is ready to emerge.

RELATION OF THE VENTRAL HEAD GLANDS TO THE PLEUROPODIA

When the locust embryo has completed its development, it is faced with the problem of breaking through the serosal cuticle and outer chorion. The latter, however, has usually dried and will often flake off readily by the time the embryo is about to

emerge. The serosal cuticle, on the other hand, remains intact but its inner layer, namely the white cuticle, is digested leaving an outer thin yellow cuticle.

Slifer (1937) demonstrated on *Melanoplus* that active principles responsible for the dissolution of the white cuticle originated from a pair of glandular organs, namely the pleuropodia, present on the first abdominal segment. For instance, if these glands were removed before digestion occurred, the cuticle remained thick and tough enough to prevent the embryo escaping. This responsibility of enzymes for the dissolution of the serosal cuticle suggests that the process corresponds to the secretion of enzymes by the epidermis for the purpose of digesting the cuticle during moulting.

In the experiments so far considered it was found that the factor released by the ventral head glands initiated moulting and that its continued secretion was necessary for further progress in development. Since it was noticed that digestion of the serosal cuticle occurred after the moult had taken place, the question arose of whether the ventral head gland factor activates the pleuropodia. A series of experiments suggests that this is the case.

When embryos were ligatured between the thorax and abdomen before the critical period for the moult, the abdomen, unlike the head and thorax, failed either to moult or show any signs of further development during the following 10 days (Text-fig. $2A_1$). Moreover, the serosal cuticle of both halves of the ligatured egg remained tough and thick (Text-fig. $2A_2$). It is to be presumed that the pleuropodia remained inactive.

As recorded earlier in this paper, a ligature between the thorax and abdomen immediately after the critical period for the release of the ventral head gland factor resulted in a moult and further but limited progress in development in the abdomen. In the case of embryos ligatured in this manner the pleuropodia were activated sufficiently to produce a differential effect upon the serosal cuticle (Text-fig. $2B_1$). The cuticle in close proximity to the pleuropodia had been partially digested, but it became progressively thicker towards the posterior end where it had retained its normal thickness of about 0·05 mm. (Text-fig. $2B_2$).

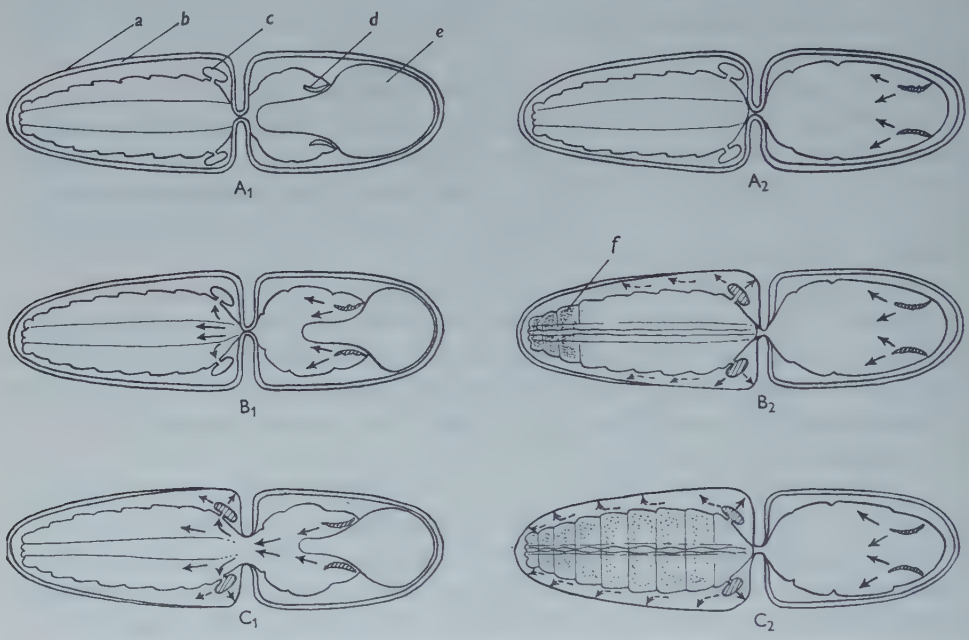
From these results it would appear that the pleuropodia, after being activated initially by the factor of the ventral head glands, were unable to secrete enough enzymes into the fluid surrounding the abdomen to digest the entire inner white layer of the serosal cuticle of the posterior half of the egg.

On this view, the inner white layer should be completely digested if sufficient factor were absorbed by the pleuropodia. This possibility was tested experimentally.

Eggs were partially ligatured to compress the embryo between the thorax and abdomen. If they were left in this condition the pleuropodia became sufficiently activated to cause the dissolution of the entire white layer of the serosal cuticle of the posterior half of the egg. In some cases in which eggs were ligatured in this manner the serosal cuticle just in front of the ligature was digested signifying a diffusion of some of the active secretion between the embryo and the serosal cuticle pressed against it. However, when the ligature was tightened 1 day after the critical period, so as to separate the abdomen completely, the pleuropodia were still unable

to bring about the digestion of the entire white part of the serosal cuticle of the posterior half of the egg. Complete ligaturing 3 days later did not prevent the entire white layer being digested (Text-fig. 2 C_1, C_2).

Accordingly, it was concluded from the preceding evidence that the pleuropodia were not only activated by the ventral head gland factor, but needed the continued secretion of the factor for about 3 days after the critical period in order to perform their function fully.



Text-fig. 2. Diagrammatic drawings of ligatured eggs of *Locusta migratoria* to illustrate the effect of dividing the embryo between the thorax and abdomen into blood-tight compartments before and after the critical period for the liberation of the development hormone by the ventral head glands to take effect upon the pleuropodia. A_1: ligatured before the critical period when both head glands and pleuropodia were inactive. A_2 shows that 8 days later the pleuropodia were still inactive. B_1: ligatured just after the critical period when head glands were active. B_2 shows that 8 days later the serosal cuticle was only partially dissolved indicating that the pleuropodia had not been made fully functional. Deposition of melanin restricted to the tip of the abdomen. C_1: partial ligature. C_2 shows that complete ligaturing at the end of 3 days of being partially ligatured after the critical period did not prevent the inner white layer of the serosal cuticle being completely digested. *a*, outer yellow layer of serosal cuticle; *b*, inner white layer of serosal cuticle; *c*, pleuropodium; *d*, ventral head gland; *e*, yolk; *f*, pigmentation. Cross-hatching signifies activity. Arrows denote flow of active principles.

RELATION OF THE VENTRAL HEAD GLANDS TO THE DEPOSITION OF MELANIN PIGMENTS

It will be apparent already that the ventral head glands exert control over events in late embryogenesis. Attention was next focused on the possibility that the deposition and spread of melanin pigment may also depend on a continued secretory activity of the ventral head glands. As may be observed in Table 1, pigmentation

of the embryo, eye-disk excepted, began after the critical period for the liberation of the development hormone. The tip of the abdomen and the distal segments of the appendages were the first parts to turn brown. Thereafter, the deposition of pigment spread gradually from these sites to form a characteristic pattern over the surface of the embryo.

Table 2 illustrates the results of ligaturing embryos between the thorax and abdomen at different times in relation to the critical period for the liberation of the hormone. It is evident from these experiments that the forward spread of melanin along the segments of the abdomen appears to depend on how long the abdomen has been in communication with the head and thorax. Consequently, it may be suggested that the amount of melanin deposited in the abdomen is proportional to the amount of hormone which has diffused in before the ligature is applied.

Table 2. *Effect of ligaturing embryos between the thorax and the abdomen upon the deposition of melanin pigments in the abdominal segments*

Time when ligature was applied in relation to the critical period for the liberation of the development hormone	Number of experiments	Abdominal segments									
		1	2	3	4	5	6	7	8	9	10
Before	34	o	o	o	o	o	o	o	o	o	o
Immediately after	26	o	o	o	o	o	o	o	+	+	+
One day after	6	o	o	o	o	o	+	+	+	+	+
Two days after	4	o	o	o	o	+	+	+	+	+	+
Three days after	12	o	+	+	+	+	+	+	+	+	+

\+ Denotes deposition of melanin.

It is generally recognized that tyrosine is oxidized in the presence of tyrosinase to form dihydroxyphenyl alanine (Dopa). Dopa is in turn changed into a polymerized product of an indole type termed melanin which is insoluble and remains where it is formed in the cuticle (Roeder, 1953). Fukuda (1956) has suggested steps in the formation of melanin beginning with plenylalanine as the original substrate.

Though melanin was not deposited until the ventral head glands had become active, it was of interest that both tyrosinase and the substrate necessary for the formation of melanin were present in the egg beforehand. If the fluid surrounding the embryo was extracted from the egg, just at the time when the embryo was revolving around the pole, it was significant that, after some delay, brown melanin was formed. Dilution of the fluid with Ringer's solution reduced this delay. On the addition of ammoniacal silver nitrate, the fluid showed a strong argentaffin reaction thus confirming the presence of dihydroxyphenols. Wigglesworth (1948) has previously demonstrated the value of the argentaffin test in his work on the insect cuticle. It was also of interest that squash preparations of embryos, at the stage of revolving, turned slightly brown and the addition of silver solution produced a relatively weak argentaffin reaction. These findings are in agreement with those of Bodine & Boell (1935) who demonstrated that tyrosinase was present in the yolk and the fluid surrounding the embryo, the amount of enzyme in the embryo itself

being very slight. However, when the embryo has reached the length of the egg, most of the fluid has been reabsorbed and all the yolk is contained in the prospective gut. Thus, at this stage in embryonic development a gradual transfer of enzyme from the fluid and yolk to the embryonic cells must occur. Though the nature of this transfer may be unknown, it is evident that the mechanism responsible for malanin formation is present and active in the embryo immediately after the moult has taken place.

DISCUSSION

The results of the present work show that the hormone released by the ventral head glands initiates the embryonic moult and subsequent progress in development up till the time the locust emerges from the egg. The critical period for the liberation of this development hormone is reached when the embryo is about three-quarters the length of the egg. Notwithstanding a subsequent reduction in the size of the glands after the moult (Jones, 1953), further progress in development requires their continued secretory activity. This conclusion was supported by the results of experiments which showed that in embryos ligatured between the thorax and abdomen, the advance in development of the abdomen depended on how long a time after the critical period the ligature was applied.

In the case of embryos ligatured just after the critical period, for instance, the abdomen moulted and showed signs of restricted development before morphogenesis came to a halt. In the case of embryos ligatured 3 days after the critical period, the stage in development reached by the separated abdomen more or less corresponded to that reached normally by an embryo within a day or so of hatching.

The conclusion to be drawn from these findings is that the completion of events in embryonic development following the moult depends upon the amount of development hormone secreted by the ventral head glands. The threshold would appear to be reached within 3 days after the moult. Accordingly, the previous suggestion (Jones, 1956) that the glands may be exclusively concerned with the embryonic moult has to be extended to include the direct control of cellular growth and differentiation after the moult. In agreement with the findings of Williams (1952) on the silkworm, *Cecropia*, the present results also indicate that this development factor is absorbed by the tissues and reacts with them to induce progress in development.

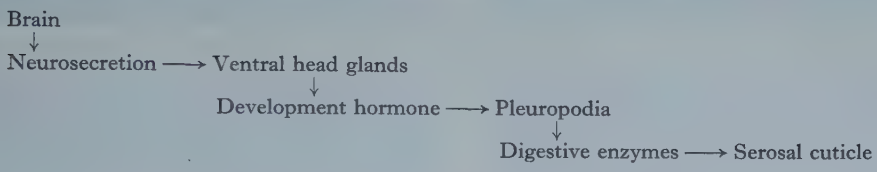
This principle of continued secretory activity of the ventral head glands as being necessary for further development after the moult may be extended to the activation of the pleuropodia. For the latter are not only stimulated by the development hormone but depend on a continued supply of this factor for a period after the moult in order to produce enough enzymes to bring about the digestion of the serosal cuticle.

When embryos were ligatured between the thorax and abdomen before the critical period for the liberation of the development hormone the abdomen failed to develop further. As a consequence the pleuropodia remained inactive as shown by the fact that the serosal cuticle surrounding the abdomen remained intact. In the case of embryos ligatured in the same manner just after the critical period the abdomen moulted and developed further. The surrounding serosal cuticle was

partially digested, suggesting that the pleuropodia had been activated but had failed to produce sufficient enzymes to complete the digestion of the inner white layer of the serosal cuticle. However, in the case of embryos ligatured 3 days after the critical period the pleuropodia were activated sufficiently to bring about the digestion of the entire white layer of the serosal cuticle.

The pleuropodia may thus be activated when the level of concentration of the development hormone present is sufficient to induce the moult. However, in order to become fully functional they depend on the continued secretory activity of the ventral head glands for a few days after the moult.

Since there is clear evidence that moulting glands are activated by a brain factor (Williams, 1952) the chain of events leading up to the digestion of the serosal cuticle in locust embryos may be thought of in general terms, as follows:



One of the most remarkable processes in insect development is the gradual spread of melanin over the surface to form a characteristic pattern. In the locust embryo the deposition of melanin is initiated after the moult has taken place, and its gradual spread continues up till the time the embryo is ready to emerge.

Pigmentation in animals has been intensively studied and that the reaction is usually an enzymic one appears well established. Indeed, the presence of the enzyme concerned, namely tyrosinase, has been demonstrated in the egg of the grasshopper, *Melanoplus differentiatis*. Moreover, the enzyme was found to be mainly confined to the yolk and extra-embryonic fluid, the amount in the embryo being relatively very slight (Bodine & Boell, 1935). The results of tests on locust eggs in the present work were in agreement with these findings. When the fluid surrounding the embryo was extracted prior to the development of the ventral head glands, it turned brown owing to the formation of melanin. The fluid also showed a strong argentaffin reaction when ammoniacal silver nitrate was added.

It is therefore suggested that in the intact egg, prior to the release of the development hormone by the ventral head glands, the enzyme-substrate system responsible for the formation of melanin must normally be inhibited. The manner of both the inhibition and liberation of tyrosinase activity is a complex problem outside the scope of the present paper. Dennell (1949) has attempted to shed some light on this particular problem.

However, the important point that calls for comment in the present work is that the development hormone appears to activate the tyrosinase-substrate system in the locust embryo. Furthermore, the system is kept active by the continued secretion of the development hormone.

Though an essentially physiological differentiation centre present in the presumptive prothoracic region of the germ band may control very early embryogenesis,

it is clear, judging by the experimental results of this work, that late embryogenesis from the time of the moult onwards is controlled by an organized endocrine system.

SUMMARY

1. Secretory activity of the ventral head glands in embryos of *Locustana pardalina* and *Locusta migratoria* is necessary for inducing the moult and controlling subsequent events in late development.

2. Continued secretory activity of the glands for a period after the moult is required for further progress in development.

3. The development hormone released by the glands activates in turn the pleuropodial glands. The latter, in order to become fully functional, also require the continued secretory activity of the endocrine glands for a period after the moult.

4. The enzyme-substrate system responsible for the formation of melanin does not become active until the development hormone is liberated. However, the system was present in the egg beforehand in an inactive state and it is suggested that it is held in check by an inhibiting influence.

5. The amount of melanin deposited appears to be correlated with the amount of development hormone released.

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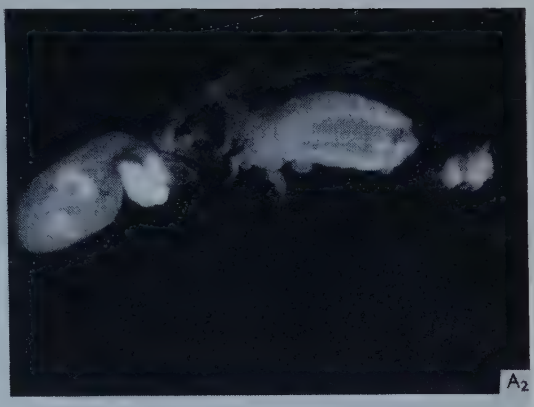
EXPLANATION OF PLATE 10

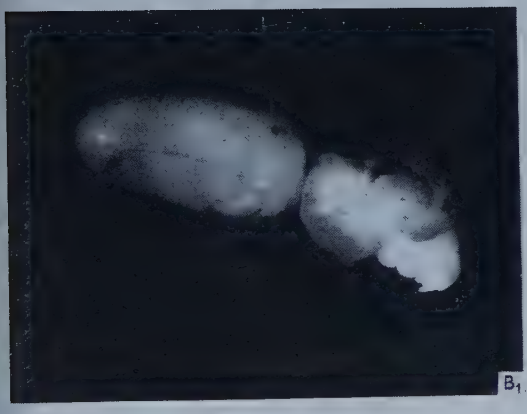
Photomicrographs of embryos of *Locusta migratoria* taken 8 days after being ligatured *in situ* at different levels before and after the critical period for the liberation of the development hormone. A and B were ligatured before and C was ligatured after the critical period. A was ligatured between the head and thorax, and B and C between thorax and abdomen.

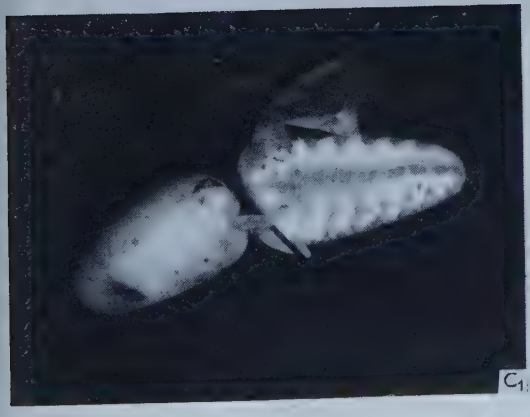
A_1: thorax and abdomen exposed. Body did not moult. A_2: hindgut relatively short, Malpighian tubules small and few, and space along mid-dorsal line is wide indicating no advance in development of the abdomen since the ligature was applied.

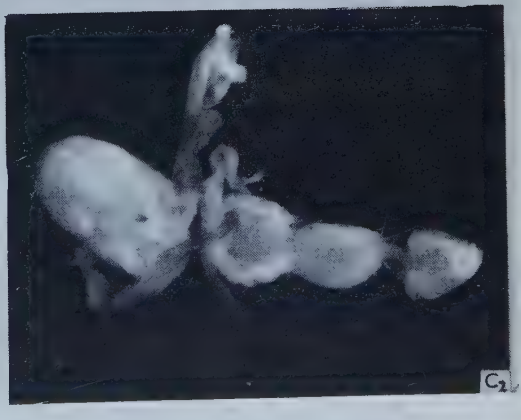
B_1: head and thorax fully occupy the anterior compartment of the egg. Abdomen has failed to moult. B_2: state of hindgut and Malpighian tubules as in A_2.

C_1: abdomen has moulted and the heart is shown as a thin tube along its mid-dorsal line. C_2: shows rounded midgut, relatively long hindgut and thread-like Malpighian tubules indicating a marked advance in development of the abdomen.









JONES—ENDOCRINE ACTIVITY DURING INSECT EMBRYOGENESIS. CONTROL OF EVENTS IN DEVELOPMENT FOLLOWING THE EMBRYONIC MOULT (*LOCUSTA MIGRATORIA* AND *LOCUSTANA PARDALINA*, ORTHOPTERA)

(*Facing p.* 696)

EXCRETION BY THE MALPIGHIAN TUBULES OF THE STICK INSECT, *DIXIPPUS MOROSUS* (ORTHOPTERA, PHASMIDAE): CALCIUM, MAGNESIUM, CHLORIDE, PHOSPHATE AND HYDROGEN IONS

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(*Received* 14 *June* 1956)

INTRODUCTION

In earlier papers (Ramsay, 1954, 1955*b*) an account was given of the excretion of sodium, potassium and water by the Malpighian tubules of the stick insect. The present paper describes the extension of this work to the more important of the remaining inorganic substances: calcium, magnesium, chloride, phosphate and hydrogen ions. An indication of the manner in which the first four are excreted is given by the analyses reported in another paper (Ramsay, 1955*a*) from which Table 1 is taken. It is seen that the concentration of phosphate is greater and the concentrations of calcium, magnesium and chloride are less in the urine than in the haemolymph or serum. (Serum is haemolymph from which most of the protein has been removed by heat-coagulation.) These analyses were made upon urine excreted by tubules bathed in haemolymph with the various components of the haemolymph at about their normal levels of concentration. It was felt that for the sake of completeness some investigation should be made of the excretion of these substances over a range of concentration.

Malpighian tubules will not survive in any known artificial medium, and hitherto it has been necessary to add serum in the proportion of 1 part to 3 parts of artificial medium. This requirement for the use of serum introduces two practical difficulties. The first is that the limitation upon dilutions greater than 1 part to 3 sets a limit to the changes in concentration of any given component which can be brought about by mixing serum with isotonic salines. This difficulty has now been overcome, as will presently be described. The second difficulty is that chronic shortage of serum makes it necessary to work with single tubules isolated in small drops of medium; and this in turn has made it necessary to adapt methods of analysis to volumes of the order of 0·1 mm.^{3} or less.

MATERIAL AND METHODS

All the results described in this paper have been obtained from the superior tubules (Ramsay, 1955*a*) of the adult female stick insect fed on privet. The preparation of the tubule isolated in a drop of medium under liquid paraffin and the collection of urine from it are described elsewhere (Ramsay, 1954).

No progress whatever has been made with the problem of separating from serum the 'active principle'—whatever it be—that enables the Malpighian tubules to function. Fortunately it has been found possible to replace the original inorganic ions (see Table 1) with others, using the method of electromigration, since the 'active principle' appears to be uncharged. The apparatus is shown in Fig. 1. It is constructed of Perspex, the main considerations in design being to combine the maximum rate of electromigration with the minimum mixing by convection and diffusion.

Table 1

	Haemolymph	Serum	Urine
pH	—	6·6	6·8–7·5
Δ	160 mM./l. NaCl	171 mM./l. NaCl	171 mM./l. NaCl
Na	8·7 m.equiv./l.	11 m.equiv./l.	5 m. equiv./l.
K	27·5 m.equiv./l.	18 m.equiv./l.	145 m. equiv./l.
Ca	16·2 m.equiv./l.	7 m.equiv./l.	2 m. equiv./l.
Mg	145 m.equiv./l.	108 m.equiv./l.	18 m. equiv./l.
Cl	93 m.equiv./l.	87 m.equiv./l.	65 m. equiv./l.
PO_4^{3-}	120 m.equiv./l.	39 m.equiv./l.	51 m. equiv./l.
Uric acid	10·4 mg./100 ml.	4·5 mg./100 ml.	43 mg./100 ml.

Note. Phosphate was expressed as trivalent is this table to emphasize the anion deficit. It is more probably present as monovalent and divalent ions in accordance with pH, from which it can be calculated that there will be 19 m.equiv./l. in serum and 30 m. equiv./l. in urine at pH 7·2.

The saline solution, say, 150 mM./l. NaCl, which is to replace the ions present in the serum, is set in agar in two tubes *a* and *b*, $\frac{3}{8}$ in. internal diameter, which connect with the two electrode vessels *c* and *d*. A continuous flow of 150 mM./l. NaCl through these vessels serves to remove the products of electrolysis, and as a check on this indicators are added to the agar in the tubes. The sample of serum, about 1 ml. in volume, is placed in the central chamber *e*. If the serum is set in agar the 'active principle' cannot be recovered, and it is therefore necessary to take other steps to reduce convection. Cellulose pulp, made from filter-paper in a Waring blendor, is squeezed dry and enough is added to the serum to make a thick pulp which will not separate out under gravity. This pulp is then placed in *e* and the whole apparatus is immersed in an ice-bath since the limit upon the current (60 mA.) is its heating effect which tends to melt the agar. After the current has passed for about 1 hr. the pulp is removed to a glass syringe and the serum is squeezed out. Replacement of ions by this method is shown by analysis to be about 95 % effective. In this way the following sera were prepared: 150 mM./l. NaC. serum; 100 mM./l. $CaCl_2$ serum; 100 mM./l. $MgCl_2$ serum; 100 mM./l. Na_2HPO_4–NaH_2PO_4 serum. Osmotic pressure and pH were adjusted if necessary to normal values. It is of course impossible to vary the concentration of one ion without at the same time varying the concentration of another. The method adopted was to study calcium and magnesium when replacing sodium, and phosphate when replacing chloride. Details are given below in the appropriate context.

The methods of analysis used were as follows. Calcium, precipitation (twice) as oxalate and titration with ceric sulphate (Kirk, 1950) and labelling with ^{45}Ca.

Chloride; electrometric titration (Ramsay, Brown & Croghan, 1955). Phosphate; ammonium molybdate method as described by Delory (1949) and labelling with ^{32}P. In the case of magnesium it was hoped to make use of ^{28}Mg but it did not prove possible to prepare this isotope free from poisonous impurities. The method eventually used was to precipitate the magnesium as magnesium ammonium phosphate using ^{32}P.

The precipitation is carried out in a droplet resting upon a cover-slip under liquid paraffin and if the conditions are suitably adjusted the precipitate separates as crystalline needles. The mother liquor can be sucked away almost completely and the cover-slip is then heated to drive off the liquid paraffin before being counted in the usual way. This method is simple and convenient but cannot be used in the presence of calcium or phosphate.

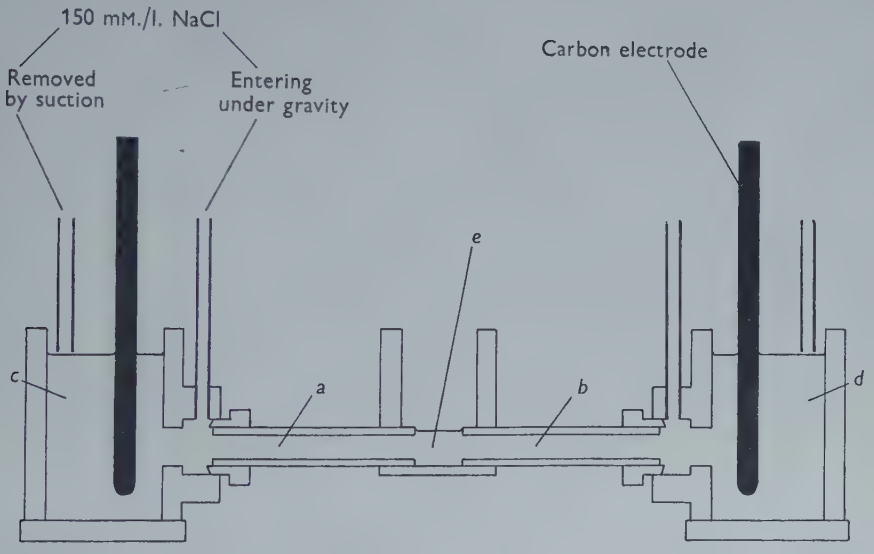

Fig. 1. Apparatus for changing the ionic content of serum by electromigration; in the illustration the ions of serum are to be replaced by 150 mM./l. NaCl. *a*, *b*, tubes containing 150 mM./l. NaCl set in agar; *c*, *d*, electrode vessels flushed out with 150 mM./l.; *e*, vessel containing serum in cellulose pulp. For further explanation, see text.

Measurements of the rate of urine production were also made, the volume of urine being found from the diameter of the droplet sinking through liquid paraffin.

It would be difficult to give precise figures for the accuracies of the foregoing methods without endless qualification as to conditions. It should be sufficient to state that the errors are nowhere greater than $\pm 10\%$ and that this degree of accuracy is sufficient to support the conclusions which are drawn from the results.

The measurement of pH presented the greatest difficulty. The electrode system most readily adaptable to small volumes is the quinhydrone electrode. Pierce & Montgomery (1935) developed a method capable of working on 0·1 mm.^{3} which gave very close agreement when compared with the large-scale method, but their pH values do not seem to have been checked against any other electrode system.

The quinhydrone electrode is of course notoriously unreliable in the presence of substances such as proteins which react with quinone, and under such conditions a stable potential is not obtained. In the present work in which the pH of serum had to be measured the drift of potential was slow and it was possible to achieve consistency by taking measurements at a fixed time after the addition of the quinhydrone. Checks were made with a conventional glass electrode whenever possible, e.g. on stock serum. Attempts were also made to develop a small-scale glass electrode. A glass electrode system capable of being used with 5 mm.^{3} of fluid

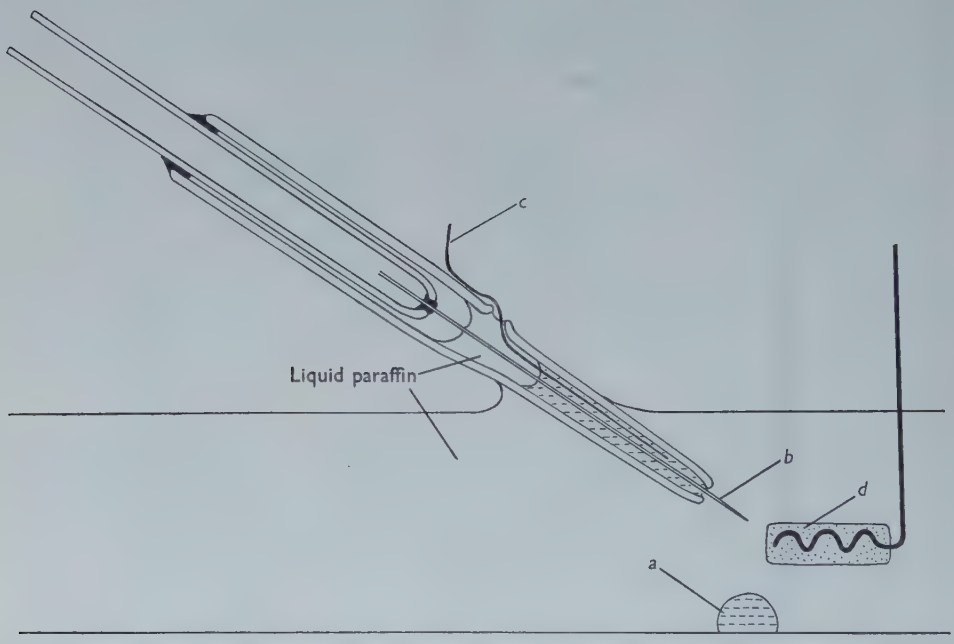

Fig. 2. Small-scale glass electrode. The droplet of solution to be tested, *a*, is kept under liquid paraffin. Some of this solution is sucked up to fill the capillary tube *b* which is of Corning 015 electrode glass. The capillary is surrounded with N-HCl with which electrical contact is made through the silver wire *c*. The tip of the capillary is allowed to come into contact with the reference electrode *d* which is a silver wire embedded in a cake of saturated KCl-agar. This electrode has been used with a Pye Universal pH meter.

has recently been described by Bishop, Hartree & McConachie (1956); the electrode is in the form of a sealed capillary which dips into a wider capillary containing the solution to be tested. Combining the capillary technique of these authors with the arrangement devised by Hartree (1952) in which the solution to be tested is inside the capillary and the standard acid is outside, an electrode suitable for working under liquid paraffin was designed (see Fig. 2). One advantage of this arrangement is that the oil does not allow the surface of the glass to develop leakage paths whereby the potential of the electrode might be short circuited. Several electrodes of this type were prepared, of from 0·3 to 0·5 mm.^{3} capacity, but (as is the usual experience) very few were of sufficiently low resistance and only one fully retained

its calibration when retested after a period of use; but this particular electrode provided an important check upon the pH of urine of which there was insufficient for use with a conventional glass electrode. For routine measurements the quinhydrone system was used throughout. A platinum wire 0·005 in. in diameter and an Ag/AgCl capillary reference electrode were inserted into the droplet to be tested —about 0·2–0·5 mm.^{3}—under liquid paraffin. Crystals of quinhydrone were then added with a glass needle and the potential was read 2 min. later. The measurements of pH are believed to be accurate to $\pm 0{\cdot}2$ pH.

All experiments were carried out at toom temperature 14–17° C.

RESULTS

(1) *Phosphate/chloride.* 1 mc. of carrier-free ^{32}P as orthophosphate was added to 0·5 ml. of Na_2HPO_4–NaH_2PO_4 serum. (The amount of phosphate thus added was less than 1 % of that already present.) KCl and $CaCl_2$ were then added to this serum and to NaCl serum so as to bring the concentrations in each to 15 m.equiv./l. of potassium and 8 m.equiv./l. of calcium. Drops of these two sera mixed in varying proportions were then set out under liquid paraffin, tubules were prepared and collections of urine were made. The results of analysis are given in Table 2 and Fig. 3A. The pH of the urine was not measured in these experiments and there is some evidence to suggest that it was affected by the phosphate content of the urine; for this reason concentrations of phosphate are expressed not as m.equiv./l. but as mg.-atoms P/l. since the valence of the phosphate in the urine is not known. From the table and figure it can be seen that the concentration of phosphate is greater and the concentration of chloride is less in the urine than in the

Table 2

Medium		Urine		Rate	
Phosphate (mg.-atoms P/l.)	Chloride (m.equiv./l.)	Phosphate (mg.-atoms P/l.)	Chloride (m.equiv./l.)	$mm.^3 \times 10^{-3}$/min.	Averages
6	118	24	106	0·92	
9	122	25	107	1·11	
11	110	14	113	1·03	1·13
12	116	29	104	1·01	
14	116	40	92	1·58	
30	93	81	61	1·46	
31	94	79	64	0·96	1·06
32	92	57	81	0·75	
49	74	101	46	1·19	
51	77	115	35	1·43	
52	71	98	45	1·61	1·49
52	75	105	44	1·43	
54	71	112	39	1·78	
66	55	127	28	1·59	
70	54	138	23	2·06	1·68
70	56	120	31	0·93	
74	51	132	22	2·16	
84	36	140	14	2·46	
84	35	129	18	1·76	2·03
87	39	137	27	1·98	
88	35	139	13	1·91	

medium, and that this is true over the whole range of concentrations studied. It is also to be noted that as phosphate is increased at the expense of chloride in the medium there is a significent increase in the rate of urine flow.

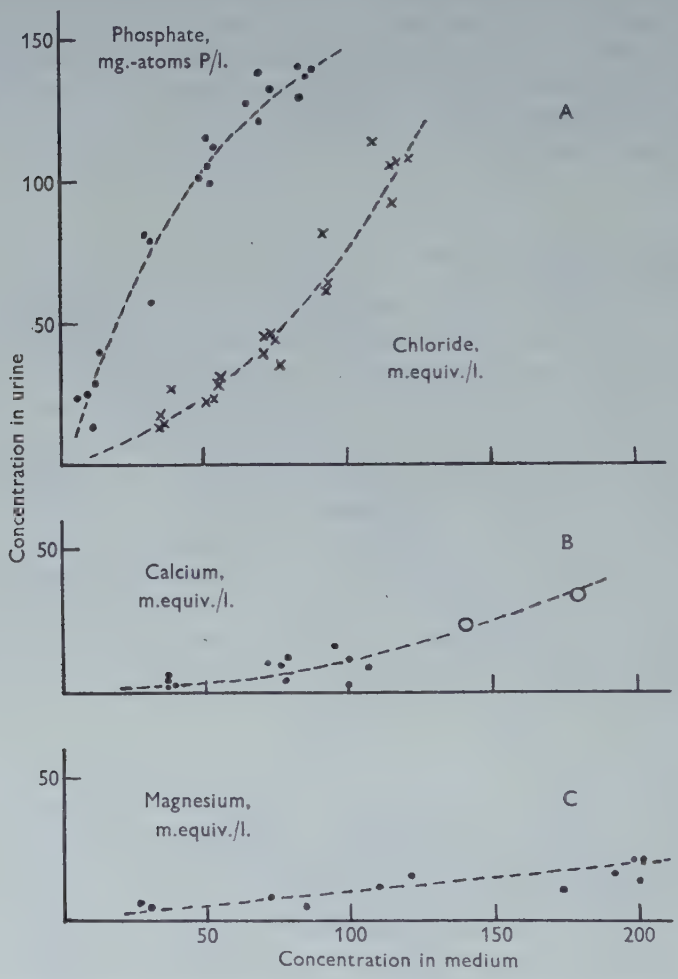

Fig. 3. The relation between concentration in urine and concentration in medium for phosphate, chloride, calcium and magnesium. Except for phosphate all concentrations are expressed as m.equiv./l. Since the pH of the urine was not measured in these experiments the valence of phosphate in the urine is uncertain, and for this reason the results have been expressed as mg.-atoms P/l.

(2) *Calcium/sodium.* 100 μc. of $^{45}CaCl_2$ (specific activity 970 μc./mg. and therefore containing about 0·1 mg. Ca) were added dry to 0·1 ml. of $CaCl_2$ serum (in which the concentration of $CaCl_2$ was about 100 mM./l. and which therefore contained about 0·4 mg. Ca). The freezing-point depression was checked and as anticipated it was necessary to add 25 mm.^{3} of distilled water to restore it to normal. KCl was then added to bring the concentration of potassium to 15 mM./l. This

labelled $CaCl_2$ serum was mixed with the NaCl serum used in the previous experiment (containing 8 m.equiv./l. of unlabelled calcium, for which correction was made). The rate of urine flow decreases as the concentration of calcium in the medium is increased, and at the higher concentrations it was necessary to pool the urine of four tubules to get enough for analysis. The results (Table 3 and Fig. 3 B) show that the calcium concentration of the urine, while increasing with the calcium concentration of the medium, is always much lower, by a factor of 5–10.

Table 3

(Concentrations in m.equiv./l. Rates in $mm.^3 \times 10^{-3}$/min.)

Calcium			Magnesium		
Medium	Urine	Rate	Medium	Urine	Rate
37	5·6	1·9	27	6.0	1·25
37	4·4	1·8	30	5·2	1·57
39	2·4	1·4	72	8·5	1·77
39	1·6	2·9	84	4·8	1·92
72	9·8	1·1	110	12·0	1·77
77	8·8	1·0	121	16·4	0·78
78	3·8	1·7	173	10·7	1·08
79	12·4	0·9	191	16·8	1·65
95	15·6	0·2	200	13·6	—
100	12·0	0·19	200	20·6	—
100	2·8	0·67	200	21·2	—
107	9·2	0·5			
*141	23·6	0·2			
*180	34·4	0·15			

* Four collections pooled.

Table 4

(Rate of urine flow in $mm.^3 \times 10^{-3}$/min.)

pH medium	pH urine	Rate	pH medium	pH urine	Rate	pH medium	pH urine	Rate
4·6	N.S.	—	6·3	7·1	—	6·5	7·1	1·7
5·0	N.S.	—	6·4	7·1	2·8	6·5	7·2	—
5·3	6·8	0·4	6·4	7·3	3·0	6·5	7·3	2·1
5·3	6·7	1·1	6·4	7·1	3·0	6·6	7·3	—
5·3	6·7	1·9	6·4	7·1	3·0	6·7	7·3	—
5·3	6·9	0·7	6·4	7·1	—	6·7	7·2	—
5·3	6·3	0·8	6·4	7·3	3·0	6·9	7·4	2·3
5·3	6·9	0·7	6·4	7·3	—	6·9	7·4	3·0
5·5	7·1	1·9	6·4	7·0	2·3	6·9	7·4	2·7
5·5	6·8	1·4	6·4	7·1	2·6	7·2	7·7	2·3
5·6	6·9	1·9	6·4	7·2	2·6	7·2	7·7	2·0
5·7	7·0	2·5	6·4	7·1	1·9	7·2	7·7	3·0
5·7	7·0	2·3	6·4	7·1	2·0	7·4	7·7	0·5
5·7	7·1	2·7	6·4	7·2	2·1	7·5	7·9	0·3
5·7	7·4	2·0	6·4	7·1	2·0	7·5	7·9	0·3
6·0	7·2	3·0	6·5	7·1	2·3	7·5	7·9	0·5
6·0	7·2	1·0	6·5	7·0	1·3	7·5	7·6	0·4
6·0	7·2	3·0	6·5	6·9	—	7·6	N.S.	—
6·3	7·2	—	6·5	7·2	—			
6·3	7·0	—	6·5	7·2	2·2			

N.S. = no secretion.

(3) *Magnesium/sodium.* The two sera mixed in this case were NaCl serum and $MgCl_2$ serum, to each of which 15 mM./l. of potassium but no calcium had been added. The results show that the concentration of magnesium in the urine is always

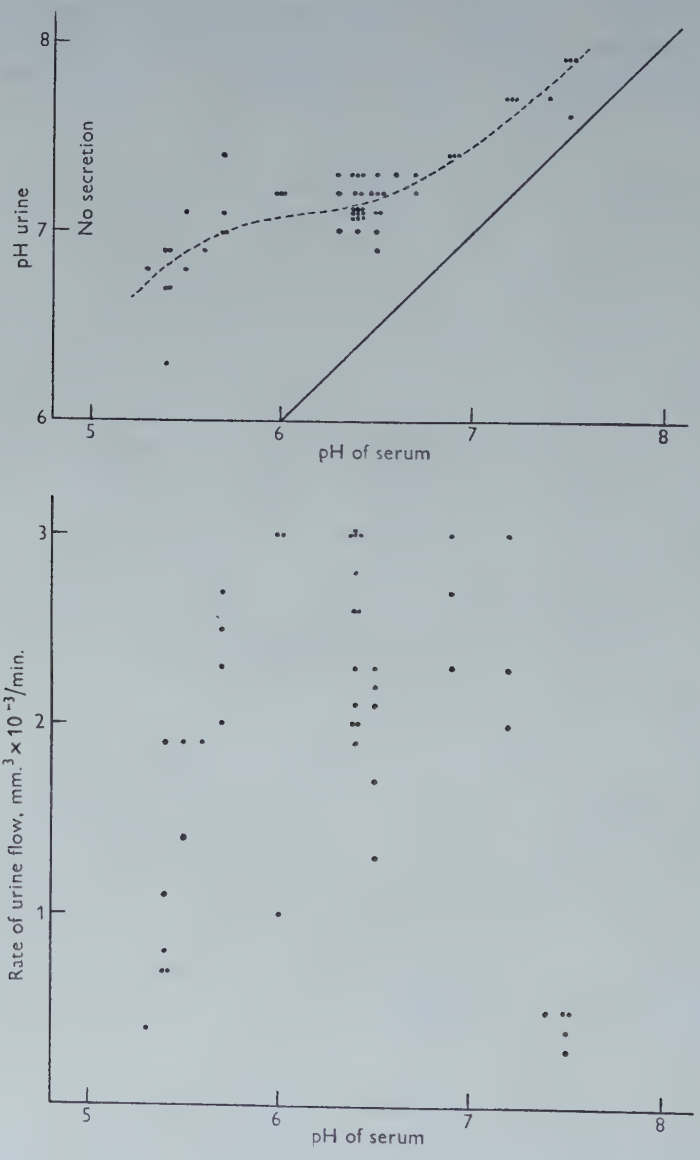

Fig. 4. The dependence of pH of urine and rate of urine flow upon the pH of serum.

low, even lower than the concentration of calcium, but high concentrations of magnesium in the medium do not seem to depress the rate of urine flow (Table 3 and Fig. 3C).

(4) *pH*. The pH of stock serum was varied by adding N-HCl or N-NaOH. The tubules were left working until a sufficient volume of urine had accumulated and a sample of serum was taken immediately after the urine had been collected; pH was then measured without delay. The results obtained in this way are presented in Table 4 and Fig. 4. The difference in pH between urine and serum is somewhat variable, but in all cases the urine is alkaline to the serum. Furthermore, the difference in pH tends to increase in the more acid media, or in other words the pH of the urine tends to remain constant in the face of changes in the pH of the medium. The measurements of the rate of urine flow show so great a scatter that it is not possible to suggest any relationship between rate and pH. All that can be noted is the decrease in rate as the working limits of pH are approached. These limits appear to be pH 5·2 and 7·5.

It is appropriate to record at this point that six measurements were made of the pH of the rectal fluid expressed through the anus. This was found to be fairly strongly acid, varying between pH 3·5 and 4·5.

DISCUSSION

Now that the scope of this investigation has been extended to include a total of seven ions the whole problem has become vastly more complex. It may be presumed that the excretion of any one ion is affected in greater or less degree by the pattern of other ions present in the medium. A fairly complete investigation has been made of the mutual interaction of sodium and potassium (Ramsay, 1955*b*); to study all possible combinations of seven ions in the same detail is hardly to be contemplated.

The present survey has done little beyond confirm what was suggested by the earlier analyses—that the concentrations of calcium, magnesium and chloride are always less in the urine than in the medium and that the concentration of phosphate is always greater. No measurements of potential difference were made during the course of this work, and it is therefore not possible to reach a definite decision as to whether energy is required for the movements of the ions now under consideration. But this does not seem likely. Calculations based upon potential difference measurements recorded in the earlier papers already referred to and upon the differences in concentration recorded here do not provide any statistically significant evidence that active transport is involved. In the case of chloride it is abundantly clear that this ion moves with and not against the electrochemical gradient; as for the others it is admissible as a working hypothesis that their movements are brought about by passive diffusion. In the case of phosphate, however, there are certain other features of the results which cannot pass without comment. Under the conditions of the experiment the concentration of phosphorus in the medium cannot exceed 100 mg.-atoms/l., whereas concentrations of up to 140 mg.-atoms/l. have been recorded for the urine. This might lead one to suppose that the osmotic pressure of the urine must have been substantially greater than that of the medium. But such does not seem to be the case; in subsidiary experiments to test this point the urine was found

to be isotonic with the medium. There are various possible explanations of this anomaly, e.g. that non-electrolytes account for a substantial fraction of the osmotic pressure of serum. There is also the possibility that some of the phosphate present is in combination with organic molecules. From his studies on the neurophysiology of the stick insect Dr D. W. Wood (personal communication) has come to this view. The excretion of phosphates merit further investigation, but it cannot be effectively studied until it is possible to prepare a fully specified medium in which the tubules will survive and secrete.

This indeed applies not only to phosphate but to the problem as a whole. As has already been mentioned no progress has been made towards isolating the 'active principle' from serum. By the methods of chromatography and ion exchange it has not so far proved possible to recover any fraction which is better than Ringer solution. A large number of substances known to be biologically active have been tested, again without positive result. One substance only, when added to Ringer, enables the tubules to preserve a healthy appearance and to excrete urine for long periods. This substance is 3-hydroxykynurenin, suggested and kindly supplied to me by Dr M. G. M. Pryor. Unfortunately it is not offered on the market and the minute supplies available from private sources would not sustain a full programme of investigation.

There being no immediate prospect of advance along these lines it may be useful at this stage briefly to review the position now reached.

It appears certain that potassium is actively transported (i.e. against an electrochemical gradient) across the wall of the tubule in the stick insect and in certain other insects (Ramsay, 1953). It is probable that this active transport of potassium is fundamental to urine production in all insects. Sodium can be actively transported but it does not seem likely that this ion or any other ion so far studied is actively transported under normal circumstances. At one time it seemed possible that the secretion of potassium (together with some anion) into the tubule would set up an osmotic pressure which in its turn would promote a passive inward diffusion of water; but having found that the osmotic pressure of the urine was slightly lower than that of the haemolymph (Ramsay, 1954) I abandoned this conception. Subsequently it was pointed out to me that since there is a potential difference across the wall of the tubule there still remains the possibility that water moves by electro-endosmosis against the slight osmotic gradient. It did not prove possible to set up the experimental conditions required to test this suggestion. The original theory is therefore still in the field, namely, that the secretion of potassium is the prime mover in generating the flow of urine and that in consequence of this secretion conditions are created which enable water and other constituents of the urine to follow.

The tubules are remarkable for their ability to continue to function in media of grossly abnormal composition. The rate of urine flow is much reduced in the presence of high concentrations of calcium, but the other ions can be varied tenfold or more in concentration without impairment of the tubule's function. There is nothing in the response of the tubules to these variations which might suggest

that they are responsible for the maintenance of the normal composition of the haemolymph; rather it is that the activity of the tubules alone would radically alter the composition of the haemolymph were it not for the participation of the other important organs of the excretory system, the rectal glands.

In his work on the tubules of *Rhodnius* Wigglesworth (1931) has proposed a mechanism for the excretion of uric acid, namely, that this substance is excreted in alkaline solution as urate into the distal region of the tubule and is precipitated as uric acid by the subsequent acidification of the urine in the proximal region of the tubule. I am not aware that there is any other insect besides *Rhodnius* in which a histological and physiological division of the tubule into two regions has been described. In the case of the stick insect there is evidence of some gradation of properties along the length of the tubule, but nothing comparable with the abrupt transition which is seen in *Rhodnius*. Furthermore, the urine issuing from the proximal end of the stick insect tubule is alkaline to the haemolymph, whereas the rectal fluid is distinctly acid. If Wigglesworth's mechanism operates in the stick insect it seems that the process of acidification must take place in the gut. In the mosquito larva which has been allowed to ingest phenol red it is observed (Ramsay, 1950) that the intestinal fluid becomes acid about 1 min. after it has reached the rectum. One is tempted to suggest that in the majority of insects the process of acidification takes place in the gut, probably in the rectum, and that the arrangement in *Rhodnius* is an adaptation for dealing with the large volumes of urine which are produced immediately after a meal of blood.

SUMMARY

1. The excretion of calcium, magnesium, chloride, phosphate and hydrogen ions has been studied in preparations of single Malpighian tubules isolated in drops of serum under liquid paraffin.

2. The concentrations of calcium, magnesium and chloride are always lower in the urine than in the serum.

3. The concentration of phosphate is always greater in the urine than in the serum. As the concentration of phosphate in the serum increases, the rate of urine flow also increases.

4. The urine is always alkaline to the serum but becomes acid in the rectum.

5. The general problem of excretion by Malpighian tubules is briefly reviewed and discussed.

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VISUAL RESPONSES BY OCTOPUS TO CRABS AND OTHER FIGURES BEFORE AND AFTER TRAINING

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INTRODUCTION AND METHOD

Octopuses are able to learn to make distinct reactions by sight to objects of different shape (see Boycott & Young, 1956). Previous experiments have mostly been devoted to training the animals by means of shocks not to attack a figure of one shape, while continuing to attack another figure that is rewarded by giving food. In the experiments reported here each octopus was first fed for several days in its home. Then a single figure was shown at a distance at successive trials and associated either with food or shock. In this way one can study the initial responses of the animals and then watch the growth of memories that are either 'positive', that is increase the probability of attack, or 'negative', if they reduce the attacks at a particular figure. The experiments were made as before (Boycott & Young, 1956) in elongated tanks $100 \times 40 \times 40$ cm., with bricks at one end, among which the octopus made its home. A figure shown at the opposite end of the tank was then made 'positive' by feeding with a dead fish if it was attacked; if there was no attack after 15 sec. a fish was introduced on a thread near to the figure. Alternatively, the figure could be made 'negative' by giving a shock if it was attacked, by electrodes attached to a holder introduced for the purpose and giving 6 V. a.c. An animal being trained to give a negative response in this way was given food at other times by dropping dead fishes into the end of the tank near the home. In order to maintain as far as possible a constant state of hunger this feeding was done at intervals throughout the day, between trials.

As a third possibility the figure shown was withdrawn if it was attacked, without giving either food or a shock. A standard time of 30 sec. was adopted for such tests, the figure being then withdrawn if there had been no attack.

The figures used were made of plastic sheet, either opaque black or opaque white, 3 mm. thick. They were attached to rods of transparent plastic, and were introduced into the tanks from above and moved up and down at a rate of two to three times a second throughout the trial.

Trials were given four times a day, with at least an hour between. Nothing was done to disturb the octopus between trials except for giving food as described above. The tanks were made of opaque asbestos sheeting. In the lid was a hole, which was covered with a wire grating between experiments. There was diffuse daylight or electric light above the tanks. The grating was removed several minutes before a

trial and the figure introduced through the hole. The octopus could therefore not see the observer and the figure appeared in the dimly lit area beneath the hole. A few of the experiments were made in tanks with open tops, where the octopus and observer could see each other, a situation with obvious disadvantages as well as advantages. The results were similar in the two types of tank.

In many of the experiments the vertical lobe system was partly interrupted either before or after training. This was usually done by cutting the superior frontal to vertical lobe tract, but it is difficult in this way to isolate the whole vertical lobe without damaging the underlying centres. The vertical lobe was therefore removed from behind in some animals, although in all cases a small portion was left intact at the front end, avoiding the possibility of damaging other centres. The extent of lesion was examined after the experiment by serial sectioning. The procedures of operation and histological examination were as described by Boycott & Young (1955, 1956).

MEMORIES PROMOTING ATTACKS ON CRABS

An octopus that has been freshly brought from the sea and placed in one of these tanks may or may not attack a crab or other figure shown a metre away, at the end distant from the home. A complicated set of factors is at work influencing the likelihood of attack; for example, disturbance during and after capture, hunger, and lack of familiarity with the tank. Animals were therefore kept for several days without the showing of any figures, fishes being given as food, in the home, six times a day.

After this period tests showed that attacks are not made equally readily by all octopuses or at all figures. For example, one octopus was given a series of tests with live crabs and various figures, shown in a random order, always with more than an hour between tests. Thus in one part of the series it was shown a black circle and waved its arms but did not leave the home; at the next trial a white horizontal rectangle, which it approached but did not attack. Then a white vertical rectangle, which was attacked, a black circle to which there was no approach at all, a white horizontal rectangle was approached, a white vertical rectangle this time elicited no approach but at the next trial a crab was shown and was attacked.

Altogether this octopus came out five times out of six (5/6) to attack crabs (which it was not allowed to catch and eat). It came out 3/6 times to attack a black rectangle (10 × 2 cm.) shown horizontally, which is a figure not unlike a large dark crab. Attacks were made 2/5 times on a white rectangle shown vertically, once each at a white and a black circle of 6 cm. diameter, but there was no attack at a black vertical rectangle or a white horizontal one. On the occasions when no attack was made within 30 sec. the octopus sometimes waved an arm at the figure from the home, or approached half way down the tank. Making a scale that awards 3 for an attack, 2 for an approach and 1 for waving the arms, the order of attractiveness of the figures appears as shown in Table 1.

It may be that other octopuses would show different orders of preference. An octopus that is being fed regularly in its home thus shows a tendency to put out the

arms to attack moving objects appearing at a distance. This tendency is not very strong and is not the same for different objects, live crabs being attacked more often than the other figures tested.

If now the animal is rewarded as a result of attacks on any figure, the attacks at this figure will become more regular and the animal will come out much more quickly. This can be demonstrated by showing crabs and allowing the octopus to eat them (Fig. 1). The octopus in this experiment attacked and ate crabs on only four out of the first ten occasions when they were shown, but then on every occasion thereafter. Moreover, the times of the attack fell off in the following manner:

19, 6, 31, 15, 24, 17, 10, 7, 10, 5, 2, 3 sec.

During the latent period before the attack is completed the octopus may sit in the home showing signs of 'attention', or may move slowly down the tank towards the moving figure (Boycott & Young, 1950, 1955).

Table 1

Figure	Attacks	Attacks + approaches, etc.
Crab	4/5	14/15
Black horizontal rectangle	3/5	12/15
White vertical rectangle	2/5	8/15
White circle	1/5	8/15
Black circle	1/5	4/15
White horizontal rectangle	0/5	4/15
Black vertical rectangle	0/5	0/15

Although all octopuses probably attack and eat crabs in the sea, they have nevertheless to learn to attack them in the particular situation in the laboratory. They bring with them a system that makes attacks on crabs more probable than on other figures, but this only becomes 'strong' enough to produce regular and rapid attacks in the new situation when the attributes of that situation have also become associated with the crab as a food object. This is not merely a matter of 'becoming used to the tank'. Octopuses that have been trained to attack some other object at the end away from the home will still not regularly or rapidly attack a crab until they have been trained to do so (p. 719).

Conversely, when an octopus has become trained to attack crabs it will not necessarily attack all figures that are shown at the same place. Thus with the octopus of Fig. 1 a white circle of 6 cm. diameter was shown at irregular intervals as a test object (no reward or shock) during the period in which crabs were being given as food (first fifty trials). The circle was attacked three times out of the first five presentations and then only twice in the next ten, although throughout this period the octopus was regularly attacking crabs. The system is therefore such that a moving object not previously associated with food may be attacked, but the probability of attack is not high and it varies from animal to animal.

In the subsequent trials in Fig. 1 food was given when the octopus attacked the white circle (or after 15 sec. if it did not) and this object rapidly became a positive

stimulus and was thereafter attacked regularly and with a short delay. Finally a second object, a circle of 2 cm. diameter, was introduced at alternate trials and shocks given when it was attacked. The attacks at this figure rapidly decreased in frequency; there was also at first a slight temporary lowering of the probability of attack on the larger circle, but then the discrimination was accurately performed.

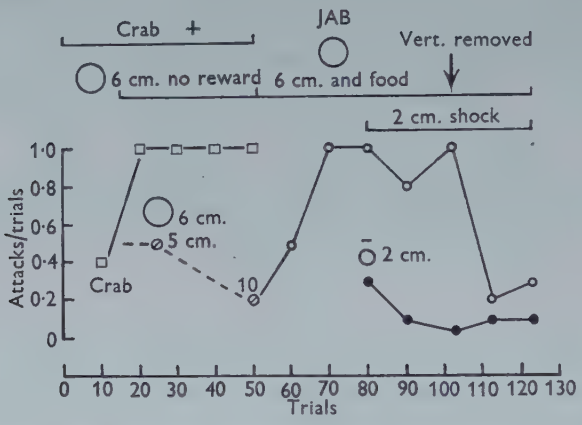

Fig. 1. For the first fifty trials the octopus was shown a crab and allowed to eat it if attacked. Beginning at the 15th trial a white 6 cm. circle was shown at irregular intervals and removed without reward if attacked. From the 50th trial onwards the crab trials were discontinued and the octopus was rewarded with fishes when it attacked the 6 cm. white circle. From the 80th trial a 2 cm. circle was introduced and shocks given if it was attacked. After the 100th trial the vertical lobe was completely removed, with some damage to the superior frontal lobe. Ordinates show the proportion of attacks in the previous ten trials, except that for the circle at the 25th trial the point represents five tests and at the 50th trial ten tests, spread irregularly over the previous period.

ATTACKS ON A FIGURE WITH NEITHER REWARD NOR SHOCKS

In the experiments of Fig. 2 a black rectangle 10 × 2 cm. was shown six times a day and was withdrawn if attacks within 30 sec. were made, no reward or shock being given. The characteristic of the responses under these conditions is that they are irregular. The octopuses sometimes came out to attack, sometimes not, and the probability of attack fluctuated in a manner not easy to correlate with any other factor. Moreover, the attacks were slow. The animals were kept in as near as possible a constant state of hunger by feeding in the home with fishes six times a day. JDD attacked more often than JDA, but in both animals there were periods of several consecutive trials in which no attacks were made. Some memory reducing the probability of attack on a given set of attributes is thus set up by the mere absence of reward.

A convenient way of expressing the results given by an octopus over a period of trials is to divide the number of attacks by the total number of showings, giving an index of response estimated over the whole period. Since the value is not stable within the period, this index is not a probability in the strict sense. It provides an index of the behaviour that an octopus has shown under given circumstances.

After an initial period of learning the animal usually settles down to a steady performance, and an index calculated over this steady period would provide an estimate of the probable further behaviour of the animal. Nevertheless, the indices used have mostly been calculated over a fixed number of trials from the beginning of any given type of training. The index then gives an expression of the behaviour of the animal available for the making of comparisons with other animals (or the same animal under different conditions), provided that the number of trials con-

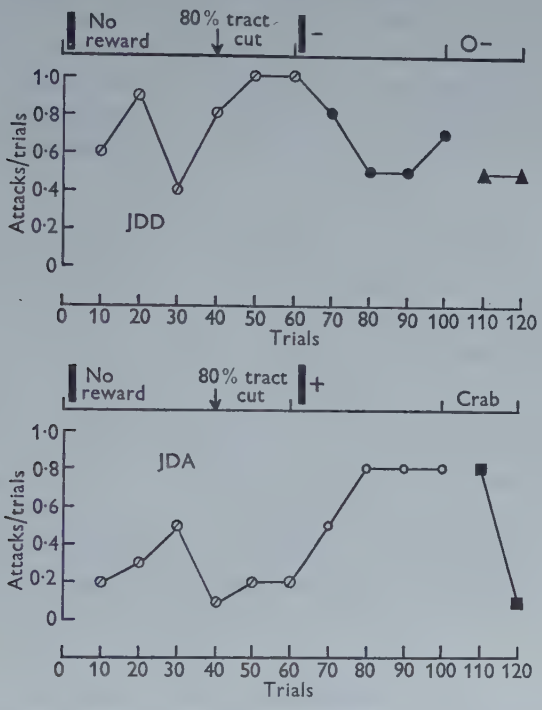

Fig. 2. During the first sixty trials a black verticle rectangle was shown and withdrawn without reward or shock if it was attacked. After the 40th trial 80% of the superior frontal to vertical tract was cut in both animals. In JDD after the 60th trial shocks were given if the black rectangle was attacked. After the 100th trial a white circle was shown and shocks given following attacks. In JDA after the 60th trial fish was given following attacks on the rectangle. After the 100th trial crabs were shown and shocks given if they were attacked.

sidered is the same. For octopus JDD this index was 0·78 and for JDA 0·28 for the parts of the experiments in which the black rectangle was shown and withdrawn without reward or shock.

After forty trials 80% of the superior frontal to vertical lobe tract was cut in these animals. The behaviour after operation was similar to that before, with a slightly greater tendency to attack, especially in octopus JDD. Every octopus has its own characteristic mannerisms and way of attacking in any given situation, for example JDA would move half way down the tank and wave its arms at the rectangle. This behaviour persisted after the operation.

It was then shown in octopus JDA that memories increasing the probability of attack can be set up after this injury to the vertical lobe. A fish was given when the rectangle was attacked (or after 15 sec. if it was not). The animal soon began to come out more regularly and quickly, but never on more than 8/10 trials, that is perhaps less regularly than in normal animals (Table 4, see p. 725). At the end of the experiment with JDA crabs were shown and the animal proved to come out readily to attack. It was not allowed to eat the crab but shocks were given following each attack. The attacks rapidly became less frequent, showing that a memory associating the crab with the shock can be built up even after removal of most of the vertical lobe. This result appears to contradict those found earlier (Boycott & Young, 1950, 1956), but the difference is that in the present experiment the octopus had never attacked and eaten crabs in the tanks. It is much more difficult to set up a memory preventing attacks if the crab has previously been made a positive figure (p. 724).

At the end of the experiment with octopus JDD shocks were given when the black rectangle was attacked and, as Fig. 2 shows, the attacks became less frequent, but still often occurred. With the vertical lobe damaged it is difficult to form a memory preventing attack on any figure. This was confirmed at the end of the experiment with octopus JDD by giving shocks when a white circle was attacked. Although this had never been a positive figure for this animal, attacks continued to be made on half the occasions. This shows clearly the value of the vertical lobe: in a normal animal a few shocks would be sufficient to prevent such attacks (p. 715).

The difference consistently shown throughout the experiments between JDA and JDD illustrates another characteristic feature. The probability of attack was always less in animal JDA than in JDD, either because of a hereditary difference or of a difference in the past experiences in the sea. Both animals were healthy throughout and readily ate the fishes given in their homes.

EFFECT OF VARIOUS PERIODS OF LEARNING TO ATTACK ON SUBSEQUENT LEARNING NOT TO ATTACK

In order to test the effect of previous 'positive' training on the ease with which a memory not to attack a given set of attributes is set up, a series of octopuses was trained for varying periods to attack a black vertical rectangle 10 × 2 cm., by giving food when it was attacked, or after 15 sec. if no attack developed. After this 'positive' training the animals were given shocks when this figure was attacked, food being now provided in the form of fishes given in the home end of the tank.

(i) *No previous positive training*

In octopus JEO, which serves as a base-line, there was no previous positive training (Table 2, and Fig. 3). The animal was isolated in the tank and fed from the beginning with fishes in its home. The black vertical rectangle was then presented at the opposite end of the tank and during the first ten trials three attacks were made and shocks received. Thereafter, during fifty further trials, spread over

8 days, there was only one further attack at this figure. Over the whole period the octopus only attacked four times, and we may therefore estimate its index of correct response as 56/60 = 0·93. It should be noted that this index is the reciprocal of that used on p. 712 to record the sequence of positive training. Tests with other figures then showed that this memory preventing attack was specific. A crab was attacked in 2/2 tests, a white 6 cm. circle in 3/3, and a black circle in 2/2. The only figure used that was not attacked was the 10 × 2 cm. black rectangle shown horizontally (0/2 tests).

Table 2. *Training not to attack a black vertical rectangle*

Octopus	Previous positive trials	Correct responses	Index of correct response	Percentage vertical lobe removed or tract cut
		A. Training before operation		
JEO	0	56/60	0·93	—
JEP	20	51/60	0·85	—
JEG	40	46/60	0·77	—
		B. Training after operation		
JFI	0	27/40	0·68	90
JFA	0	36/60	0·60	75
JFC	0	45/60	0·75	60
		C. Trained to attack before operation, not to attack after operation		
JDQ	20	39/60	0·65	50
JDB	40	46/60	0·77	40
JDC	80	35/60	0·58	75
		D. Trained to attack after operation, and then not to attack		
JDT	20	36/60	0·60	40
JDR	40	29/60	0·49	40
JDS	80	29/60	0·49	60

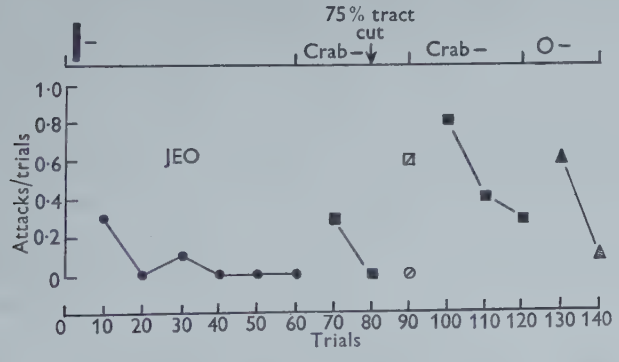

Fig. 3. Training not to attack a rectangle. For the first sixty trials a black vertical rectangle was shown and shocks were given if it was attacked. For the next twenty trials a crab was shown and shocks given following attacks. 75 % of the superior frontal to vertical lobe tract was then cut. Ten tests each were then given with the crab (▨) and black rectangle (⊘), no reward or shocks being given. From the 90th to the 120th trial crabs were shown and shocks given following attacks. From the 120th to 140th trial the white circle was shown, followed by shocks.

Over the next series of trials a crab was shown and shocks were given when it was attacked. After only three such shocks there were no further attacks in twenty trials. A memory preventing attacks on a crab is therefore readily set up if there has been no period of learning to attack crabs in this situation, and we may estimate the index of correct response as $17/20 = 0{\cdot}85$, though because of the difference in number of trials this index is not fully comparable with that for the black rectangle. Further tests showed the same conditions as before, circles being attacked, but neither the crab nor rectangle (crab 0/2, black vertical rectangle 0/2, black horizontal rectangle 0/2, white circle 2/2, black circle 2/2).

At this point 75 % of the superior frontal to vertical tract was severed. Tests showed that the memory preventing attacks on the crab had now been largely dissipated, but the tendency to attack persisted; attacks were made in 6/10 tests (without shocks). During the same period, however, the black vertical rectangle was never attacked. This shows at once the difference between the attributes of a crab and of other figures. Although crabs had never provided food in this situation they had presumably done so previously in the sea. A memory preventing attacks on them was readily set up while the vertical lobe was intact, but this was largely dissipated when the lobe was isolated. On the other hand, the memory preventing attacks at the black rectangle did not disappear after severance of the tract. This is a figure for which there was no previous memory and the representation of it in the optic lobes is held linked with the attributes of 'shock', even without the assistance of the vertical lobe. This experiment also shows that removal of the vertical lobe influence does not lead simply to a generalized tendency to attack all moving figures (see also Boycott & Young, 1956).

In the next series of trials with JEO shocks were again given when the crab was attacked. The attacks were much more persistent than before operation, the animal coming out 15/30 times, as compared with only 3/20. However, attacks became gradually less frequent, showing that a memory preventing attacks on crabs can be built up even after this injury to the vertical lobe, but that such a memory is much less rapidly established than in the normal animal and tends to dissipate, leaving an irregular performance. The index of correct response has fallen from 0·85 before operation to 0·50 afterwards.

The white circle, however, was still attacked (Fig. 3), showing that the memory partially preventing attacks on crabs was specific. Shocks given with the white circle then led to a decrease in attacks on it, the rate of learning being greater than when training not to attack crabs, but slower than the original learning by the intact animal not to attack the black rectangle. The vertical lobe therefore also plays some part in learning not to attack figures not previously associated with food.

At the end of this long series of trials associated with shocks the octopus rarely came out to attack any figure, but did not otherwise appear inhibited or withdrawn, often putting out an arm when the figure first appeared.

The above conclusions find confirmation in further experiments. Thus in another octopus (JDP) shocks were given from the beginning when a black vertical

rectangle was attacked and this figure rapidly became negative, there being in all six attacks in forty trials, four of them in the first ten. Tests then showed that crabs and white circles were still attacked, but not a black horizontal rectangle.

(ii) *Reversal after positive training*

In two normal octopuses, before giving shocks, short periods of training to attack the black rectangle were given, the animals being rewarded with fishes. Fig. 4 and Table 1 show that in octopus JEP, in which there were twenty initial

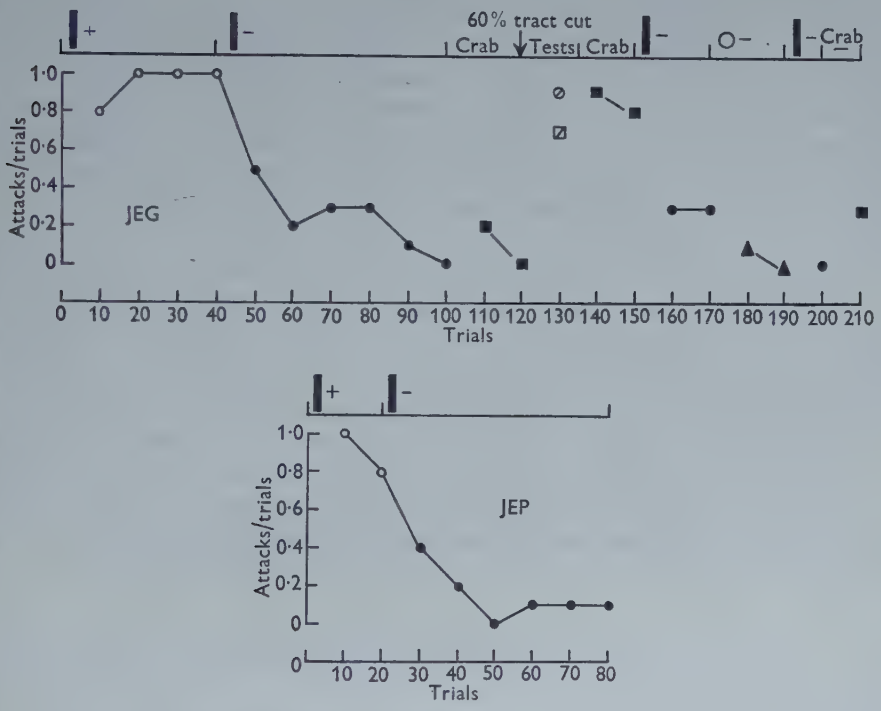

Fig. 4. Training first to attack and then not to attack a rectangle. In JEP there were twenty and in JEG forty initial trials in which fish was given when the rectangle was attacked. For the next sixty trials in both animals shocks were given after the rectangle was attacked. In JEG there were then twenty trials in which a crab was shown and shocks given if it was attacked. 60% of the superior frontal to vertical lobe tract was then cut. After ten tests each with the crab (▨) and rectangle (⊘) without reward or shock there were then twenty trials with the crab followed by shock and then twenty with the rectangle and shock. From the 170th to the 190th trial a white circle was shown and shocks given. For the next ten trials the rectangle was shown but never attacked. Finally, in the last ten trials crabs were shown and shocks given.

training trials, the black rectangle was attacked nine times in spite of shocks, in the subsequent sixty trials ($I=0{\cdot}85$). In octopus JEG, with forty initial trials, there were fourteen such attacks ($I=0{\cdot}77$). The animal JEO, with no initial 'positive' training (p. 714), only attacked four times in sixty 'negative' trials ($I=0{\cdot}93$). It is thus clearly shown that it is more difficult to set up a memory preventing attack on a figure that has been previously associated with food (see Table 2). Both JEP and

JEG, when they had learned not to attack the vertical rectangle, readily attacked crabs. Evidently the crab, although it had never provided food in this situation and had not been seen at all for 10 days, provided a strong positive stimulus.

Octopus JEG continued to attack other figures when it had finally learned not to attack the black vertical rectangle. It even attacked the same rectangle shown horizontally. The memory preventing attack can therefore be highly specific for one set of attributes.

At this stage (trial 100, Fig. 4) shocks were given when JEG attacked crabs, and a memory preventing attack was set up so rapidly that only two attacks were made in twenty trials ($I = 0{\cdot}90$). Now 60% of the vertical lobe tract was cut. Tests without reward or shock immediately thereafter showed numerous attacks on both the crab and the black vertical rectangle. In octopus JEO (Fig. 1), where the rectangle had never been a positive figure, elimination of the vertical lobe did *not* lead to attack on the rectangle. The contrast with JEG is striking and suggests that the vertical lobe plays an especially important part in maintaining memories that prevent attacks in situations that have been previously associated with food.

The setting up of a memory preventing attacks on crabs now proved nearly impossible in octopus JEG, there being attacks on 17/20 occasions ($I = 0{\cdot}15$). The black rectangle was, however, easily again made negative (6/20 attacks, $I = 0{\cdot}70$), and finally the white circle, which had never been a positive figure, was attacked and shocked only once in twenty trials. The ease with which memories preventing attack are set up by animals with reduced vertical lobe tissue thus depends upon the extent to which the figure concerned already constituted a positive stimulus. The series is uncontrolled, however, in the sense that shocks were given first with the crabs and last with the white circle. However, further tests (trials 190–210) then showed that the result was not due simply to this sequence of presentation. Crabs still proved able to elicit more attacks than any other figure.

(iii) *Setting up of memories preventing attack after operation on the vertical lobe*

(*a*) *No positive training*

In the next series the animals were operated at the beginning of the experiment, after a period of 2 days in the tank during which they were fed with fishes in the home. During this time tests were made with various figures, the animal being neither rewarded nor shocked if it attacked them (see p. 710). After operation, when the black vertical rectangle was presented, it was attacked and shocks given, the experiment being continued as usual for sixty trials. Fig. 5 and Table 2 B show that attacks were much more numerous than in intact animals. JFA made 24/60 attacks and JFI 13/40 ($I = 0{\cdot}60$ and $0{\cdot}68$). In JFA there was no improvement during the course of the experiment, attacks were made about four times in each ten trials, sometimes at several consecutive trials, with periods of no attacks sometimes as long as nine trials. At the end of the series of trials with JFA further tests showed that the octopus would usually come out to attack a crab, but would only put out an arm or approach towards other figures. Finally shocks were given when the octopus

came out to attack crabs. The animal slowly learned not to attack (see p. 724). In a third octopus, JFC (Fig. 5 and Table 2), crabs were shown immediately after operation and shocks given if they were attacked. The animal continued for sixty trials to attack on about 4/10 occasions (p. 724). In the next sixty trials the black vertical rectangle was shown and shocks given. Attacks were rather less frequent than on the crab, but still occurred on many occasions ($I=0{\cdot}75$). Memories preventing such attacks are much less reliable after these lesions than in a normal animal (Table 2).

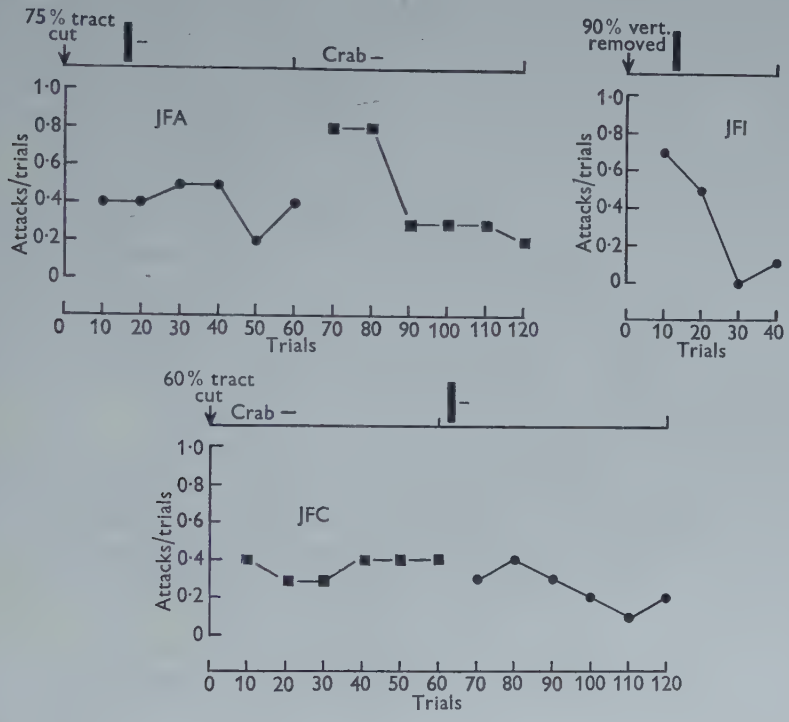

Fig. 5. Learning not to attack after vertical lobe lesions. Lesions were made at the beginning of each experiment. In JFA and JFI a black vertical rectangle was shown in the subsequent trials, shocks being given if it was attacked. JFC was trained not to attack crabs in the first sixty trials after operation. Subsequently it was trained not to attack the rectangle.

(*b*) *After positive training before operation*

Three octopuses were trained for twenty, forty and eighty trials, to attack the black vertical rectangle before operation. When this positive response had been established tests were made with crabs and other figures and these were usually attacked, but always after much longer delay than when the rectangle was shown. For example, octopus JDB, after forty trials with the black vertical rectangle, rewarded by fishes, came out regularly within 3 sec. to attack that figure. When a crab was shown the octopus remained for 10 sec. moving its head up and down in the home, then put out an arm and moved on to the top of the bricks. Waving the arms

towards the crab it moved slowly half-way down the tank and finally dashed at the crab 27 sec. after it had appeared. This is clearly a positive reaction, but not a strong one such as is given to the black vertical rectangle. The memory producing attacks at the latter was quite specific and the same rectangle, when shown horizontally, was not attacked (three trials). This demonstrates very clearly that a specific memory ensuring attack on the rectangle had been established, making it a more 'attractive' figure in this situation even than crabs, which had presumably provided food in the sea.

The vertical lobe tract was then partly cut and after this operation the octopuses continued to emerge to attack the black vertical rectangle more readily than crabs or other figures. Memories ensuring attack thus survive this operation and correct performance of attacks does not involve the integrity of the vertical lobe. Shortly after operation shocks were given when the rectangle was attacked, food being provided by giving fishes at the home end of the tank. Fig. 6 and Table 2 C shows that far more attacks were made than by the intact animals of Table 2 A. These animals in Table 2 C tended to show more attacks in the early period and there was a gradual improvement, but the total number of attacks in sixty trials is similar to that shown by JFA and the other animals in Table 1 B. Unfortunately the differences in the extent of interference with the vertical lobe system make exact comparisons difficult and do not allow us to say exactly how the amount of preoperative positive training influences the ease with which memories preventing attack on a given figure are set up without the vertical lobes.

JDQ, the animal with the least amount of positive training in this set, rapidly stopped attacking when shocks were given, but then began again for a period. Only half of the vertical lobe tract had been cut and at the end of the sixty negative trials this animal attacked the black vertical rectangle only rarely. Tests then showed regular attacks on crabs and a white 6 cm. circle, but not on a rectangle shown horizontally.

The animal was next shown to be readily made negative to both the white circle and to the crab, one shock sufficing in each case to prevent attacks for the subsequent nine trials. Even an animal without a complete vertical lobe can thus quickly form memories preventing attack on figures that are not already strongly positive. During the next twenty trials the octopus was allowed to eat crabs when it attacked them. After this period of positive training a negative memory preventing attacks on crabs was then set up only with much greater difficulty. The animal attacked seven times in the first ten trials in spite of the shocks received. After thirty trials attacks on the crab fell almost to zero, but for the whole thirty trials the index of correct response was only 0·63. Over the first ten trials it was 0·30 as against 0·90 before the positive training with the crab. Tests with the white circle and black rectangle showed attacks on nearly all occasions. The memories attached to these figures had faded and they were not involved in the specific memory preventing attacks on crabs.

Octopus JDB, with forty positive trials before operation, shows essentially the same sequence, but became 'negative' rather more easily than JDQ in spite of the

greater amount of positive training; fourteen attacks were made in sixty trials ($I = 0{\cdot}77$), only 40% of the tract had been severed. As before it was then found that slow attacks were made upon crabs or a white circle, but these were very rapidly inhibited by shocks. The white circle was then made into a positive figure by rewarding with fishes when it was attacked (or presenting them with the figure if no

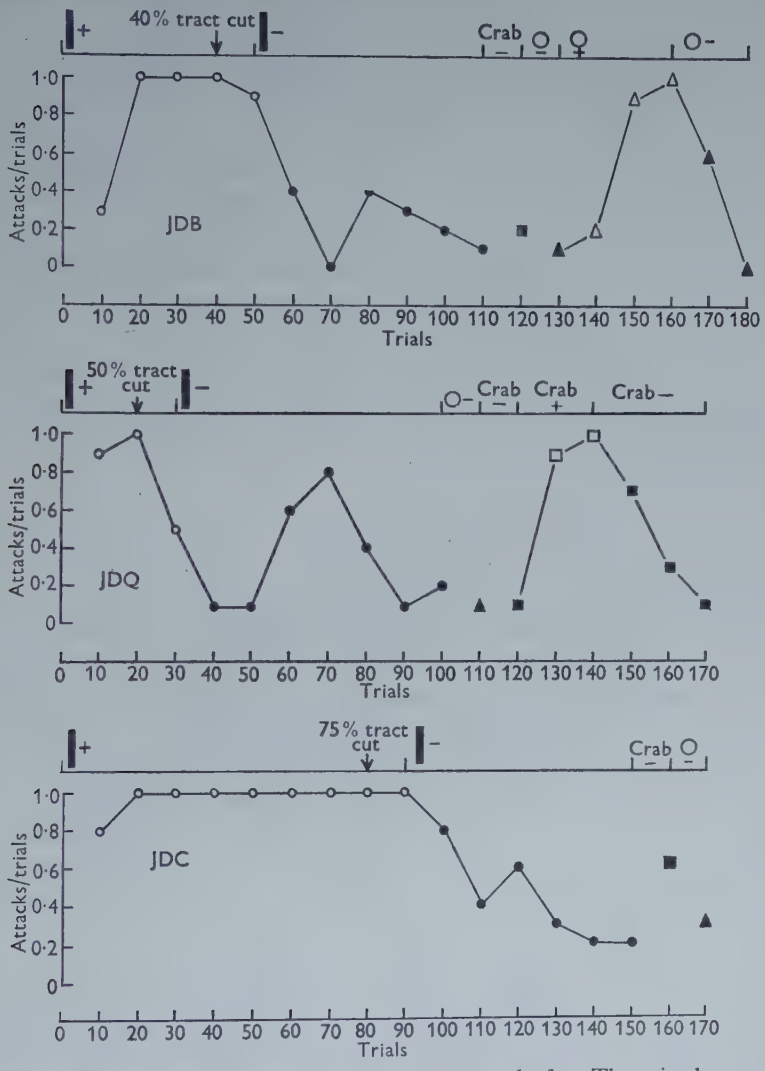

Fig. 6. Learning to attack before a lesion, and then not to attack after. The animals were given first respectively forty, sixty and eighty positive trials with reward. They were then operated and given sixty negative trials, with shocks if they attacked. JDQ was then given ten negative trials, each with a white circle and a crab. From the 120th to 140th trials it was then allowed to eat the crabs, but from here onwards shocks were again given if a crab was attacked. JDB, after the sixty negative trials with the rectangle, was given ten negative trials each with crabs and a white circle, and then from trials 130 to 160 the white circle was made positive by rewards. Finally shocks were again given for attacks on the circle. In JDC the experiment was ended by ten negative trials, each with crabs and the circle.

attack developed in 15 sec.). After thirty trials a positive response was well established (showing that the complete vertical lobe is not necessary for this). Crabs shown at this time were not attacked; the negativity attached to their attributes had persisted for 8 days, the positive factor becoming attached only to the white circle. Shocks were then given when the white circle was attacked, which occurred six times in the first ten trials, a marked contrast to the one attack in ten before positive training. However, after these six shocks the white circle was not further attacked; a memory preventing attack on it had been developed in spite of the injury to the vertical lobe. Finally tests showed that the black rectangle was now no longer a negative figure. It had not been shown for 9 days and the original positive effect attached to it had returned. Moreover, there was evidence that the specific memory of the set of attributes 'black vertical rectangle' had survived, for a rectangle shown in the horizontal position was also not attacked.

JDC was the member of this set with the greatest amount of positive training before operation (eighty trials). The memory ensuring attack survived interruption of 75% of the tract and was reversed only very slowly by shocks. There were twenty-five attacks in sixty trials, mostly in the early stages ($I = 0{\cdot}58$). There was definite improvement in performance, but mistakes were made even at the end. After this time there were attacks on crabs or a white circle; the negativity partially attached to the rectangle was specific.

(*c*) *Training to attack after section of the superior frontal to vertical tract*

In a further three animals the operation was performed as soon as they had become accustomed to the tanks and the positive training was then given (Fig. 7 and Table 4). The learning to attack was in each case rather slow, the total number of attacks in the first twenty trials for these three animals taken together being 42/60 as against 50/60 for the animals in the last section, which were trained before operation. This suggests a possible effect of the vertical lobe on the setting up of positive memories (see p. 727), but further experiments are needed. There were some signs that in the early stages of positive training the memory that ensures attack lasted only for a short time. These attacks were made more regularly at tests given an hour after previous tests than at the first trial of the day. Such 'short memories' are a conspicuous feature of learning not to attack in animals with no vertical lobes (Boycott & Young, 1955).

Tests at the end of the period of positive training showed that the octopus would come out to attack crabs or other figures, but less regularly and more slowly than to attack the rectangle. Thus in JDS the order of 'positive' effect was black vertical rectangle > crab = white circle > black horizontal rectangle.

The training not to attack then followed the same course as in the animals made positive before operation (Table 2 D). In all three octopuses it was difficult to reverse the positive tendency, even though it had been set up after operation. Indeed the number of attacks was greater in this series than in the previous one, although the lesions were smaller. Signs of the effect of the amount of positive

training appeared in the fact that the animal with only twenty positive trials attacked twenty-four times in the subsequent sixty trials ($I=0{\cdot}60$) as against thirty-one times ($I=0{\cdot}49$) for each of the animals with forty and eighty positive

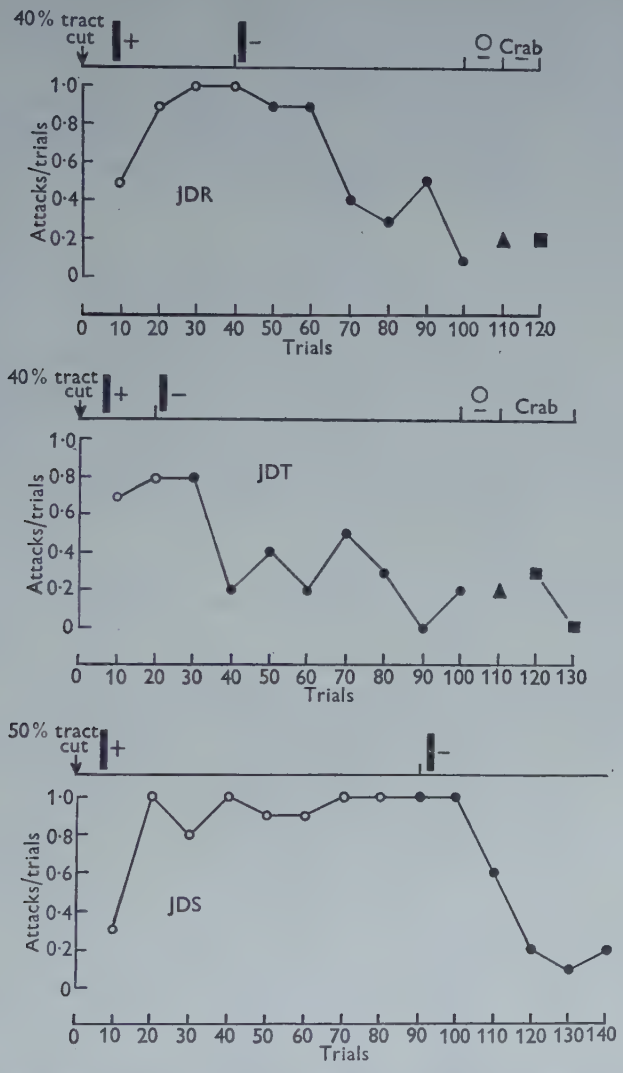

Fig. 7. Training after lesions, first to attack and then not to attack. All the animals were operated at the beginning of the experiment. They were then given twenty, forty or eighty positive trials with the rectangle followed by reward. Each was then given sixty trials in which attacks were followed by a shock. The experiments were ended by negative trials with a white circle and with crabs.

trials. The proportion of attacks given by these animals trained positively after operation was similar to that in the previous series trained before operation (Table 2 C, D). Differences in the extent of lesion make close comparison difficult, but there is a suggestion that the memories set up *after* operation are more difficult to

reverse. Thus JDS attacked at every one of the first twenty negative trials in spite of the shocks received. However, by the end of sixty trials attacks had become rare ($I = 0{\cdot}49$ for the whole period).

The outstanding feature that emerges from the four series in Table 2 is that animals with impaired vertical lobe function learn only slowly not to attack a previously positive figure, and that their performance is erratic.

At the end of sixty trials the black vertical rectangle was attacked only seldom by the animals of series 2 D, but attacks were still readily made on a white circle or on crabs. By means of shocks this positivity was then readily reversed, as in the previous series.

EFFECT OF PREVIOUS TRAINING ON SETTING UP OF MEMORIES PREVENTING ATTACKS ON CRABS

It has already been shown that a normal octopus can readily be trained *not* to attack crabs (Boycott & Young, 1955). There has been no systematic investigation of the effect of the amount of previous *positive* training on the later learning not to attack. Octopus JFG (Fig. 8) shows that with no previous positive training the memory preventing attack is quickly set up in a normal animal—there were only three

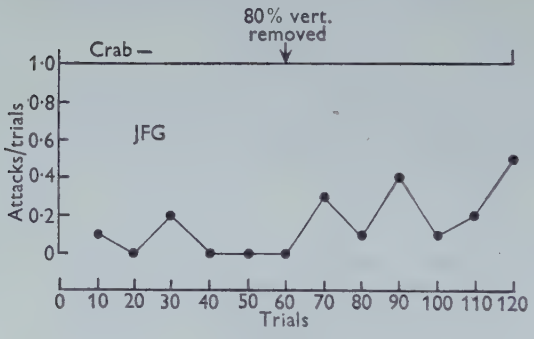

Fig. 8

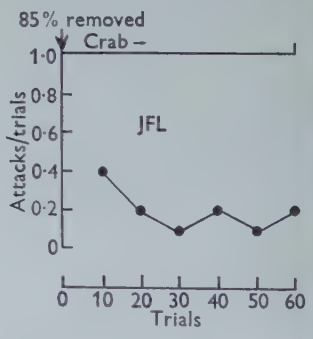

Fig. 9

Fig. 8. Octopus trained not to attack crabs for sixty trials and then 80 % of vertical lobe removed. The attacks became more frequent.

Fig. 9. 85 % of vertical lobe removed immediately the animal arrived in the laboratory and the octopus then trained not to attack crabs for sixty trials.

attacks in sixty trials ($I = 0{\cdot}97$). The vertical lobe was then removed (80 %) and the animal attacked more often, but still only 16/60 times ($I = 0{\cdot}73$). Such memories can therefore be maintained without the intact vertical lobe.

In three octopuses the vertical lobe tract was partly cut, or the lobe removed, immediately after the animal arrived in the laboratory and shocks were then given when crabs were attacked (Figs. 5, 9). Over the next sixty trials in JFC (60 % cut), there were twenty-two attacks, either three or four in each ten trials, without any systematic improvement ($I = 0{\cdot}63$). Similarly, in JFA (75 % cut) there were 27/60 attacks at the crab ($I = 0{\cdot}55$). In JFL (85 % removed) there were only 12/60

attacks ($I = 0{\cdot}80$, Fig. 9), which is still, however, more than would be made by a normal animal (Table 3).

Table 3. *Responses of octopus when shocks are given when crabs are attacked*

Octopus	No. of positive trials	Correct responses	Index of correct response	Percentage vertical lobe removed or tract cut
		A. No previous training to attack crabs		
JEO	—	17/20	0·85	0
JEG	—	18/20	0·90	0
JFG	—	57/60	0·97	0
JFA	—	48/60	0·80	85
JFG	—	44/60	0·73	80
JFA	—	33/60	0·55	75
JEO	—	15/30	0·50	75
JDC	—	4/10	0·40	75
JFC	—	38/60	0·63	60
JEG	—	3/20	0·15	60
JDQ	—	9/10	0·90	50
JDB	—	8/10	0·80	40
JDT	—	7/10	0·70	40
JDR	—	8/10	0·80	40
		B. With previous training to attack crabs		
JFB	40	33/60	0·55	70
JDQ	20	3/10	0·30	50
JDE	100	13/40	0·32	35
JED	100	35/60	0·58	20

Table 4. *Responses to positive training with black vertical rectangle*

Octopus	Attacks	Index of response	Percentage tract cut
	A. No training		
JDD	27/40	0·68	—
JDA	11/40	0·28	—
	B. With positive training		
JEP	18/20	0·90	—
JEG	38/40	0·95	—
JDQ	19/20	0·95	—
JDB	33/40	0·82	—
JDC	78/80	0·98	—
	C. With positive training after cutting tract		
JDA	31/50	0·62	80
JDR	34/40	0·85	40
JDT	15/20	0·75	40
JDS	69/80	0·86	60

In a number of other operated animals the effect of giving shocks when crabs were attacked was tested, without previous positive training. The results are summarized in Table 3, arranged in order of completeness of removal of the vertical lobe. All the animals learned more slowly than the three normal octopuses shown at the top of the table. However, there is considerable variability among the

operated group. Some of those with the larger lesions gave a fairly accurate performance and vice versa. Octopus JEG, for example, with 40% of the vertical lobe intact, attacked on nearly all occasions, giving an index of correct response of only 0·15, whereas JFL with only 15% intact gave 0·80. The explanation of these differences may lie in the history of the animals before coming to the laboratory, some having been more particularly crab-eaters than others. Evidently the vertical lobe is involved in setting up memories that prevent attacks on crabs, but it is not essential that the lobe be intact if the crab has not been learned as a positive figure in this situation.

In animals that had learned to attack crabs in the tanks it was in general more difficult to set up memories preventing attack on crabs after vertical lobe injury. Thus JFB, with 70% of the tract cut (Fig. 10), was given forty trials in which it was

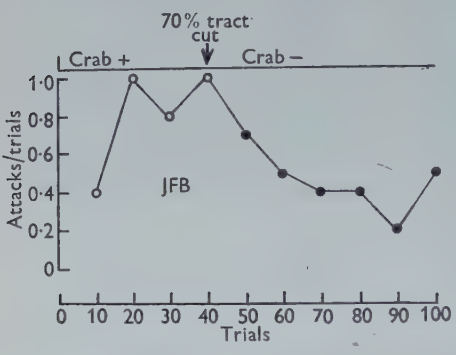

Fig. 10

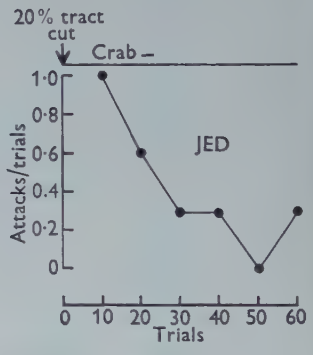

Fig. 11

Fig. 10. Octopus trained to attack crabs for forty trials and then 70% of the superior frontal to vertical lobe tract cut and the animal given shocks if crabs are attacked. There are far more attacks than in Figs. 8 and 9, where the animal had not been allowed to eat crabs before operation.

Fig. 11. The octopus had previously undergone a long series of experiments with crabs as reward after 20% of the superior frontal to vertical lobe tract had been cut. Then shocks were given when crabs were attacked.

allowed to eat the crabs and the vertical lobe was then removed. Shocks were then given whenever crabs were attacked and there were found to be 27/60 attacks ($I=0{\cdot}55$). Other animals confirmed this finding: JDQ (p. 720) gave $I=0{\cdot}30$ after twenty positive trials with crabs. Octopus JDE had been used in a long series of experiments in which crabs had been given as reward. Although only 35% of the vertical lobe tract had been cut the animal attacked on 27/40 occasions on which it was shown crabs and shocked if they were attacked ($I=0{\cdot}32$). Similarly, in octopus JED, after a long series of experiments with crabs as reward, subsequent to 20% section of the vertical lobe tract, the animal was trained not to attack crabs. It showed gradual improvement, but attacked on 25/60 occasions, $I=0{\cdot}58$ (Fig. 11). These experiments do not show precisely the effect of various amounts of positive training on the setting up of memories preventing attacks on crabs. They show that even with vertical lobes nearly intact such memories are formed only with difficulty and are liable to break down, the positive response tending to reappear. Memories

having some effect in preventing attacks on crabs can be set up after removal of most of the vertical lobe using intervals of about 2 hr. between trials. However, the operated animals behave much less 'correctly' than normal ones in this situation, and it is especially difficult to prevent attacks on crabs if the octopus has been allowed to eat these while in the tank.

DISCUSSION

Feeding an octopus in its home for some days and then testing its response to figures shown at the other end of the tank provides a useful technique for revealing the capacities that the animals bring with them from the sea. The result may be expressed by saying that they tend to attack any small figure moving in the visual field, but the probability of attack within a given time is less than one, and varies from octopus to octopus. Although there has been no very thorough investigation, the evidence is that not all figures are equally likely to be attacked; crabs and horizontal rectangles are attacked more often than other figures (p. 710), the horizontal rectangles perhaps because they resemble crabs. An octopus therefore brings from the sea a store of information associating certain visual attributes with food. The effect of this memory in ensuring attack is not very strong, but after crabs have been attacked and eaten in the tank the probability of attacks on them greatly increases. Similarly, other figures, which are at first only sometimes attacked, will be attacked regularly after they have been associated with food. Conversely, if a crab or other figure has never been associated with food in the tank, then if shocks are given when it is attacked a memory preventing attack upon it is readily set up, but this is much more difficult if the figure has become associated with food.

There is some evidence that the memory associating a given set of attributes with food resides in the optic lobes. After considerable amounts of the vertical lobes have been removed an octopus comes out to attack crabs more readily than other figures (p. 716). The neuronal system that had been previously set up representing the association of the attributes of a crab with food was not disturbed by the operation—presumably because it resides in the optic lobes. However, animals with vertical lobe functioning impaired learn the positive association between a given figure and food rather more slowly than normal (Table 4). The vertical lobe is therefore involved in the process of setting up 'positive' memories in the optic lobe, by which the attributes of a situation are associated with food. It would be interesting to repeat this experiment with removal of larger amounts of the vertical lobes.

The present series provides clear evidence that the vertical lobe plays a part in setting up memories that associate a set of attributes with a shock. This effect is seen even if the figure had never previously been associated with food. Thus the operated animals of series B in Table 2 learned not to attack a black rectangle more slowly than did JEO of series A. If a figure has been previously associated with food, then it is more difficult for an operated animal to store the information that associates it with a shock. The animals in series C and D of Table 2 learned less well than those in series B, though the difference is complicated by the variation in the amount

of tissue remaining. The vertical lobe may thus be especially involved in the setting up of memories that prevent attack on previously positive figures. The fact that after vertical lobe removal the animals tend to make mistakes in the direction of attacking in spite of shock has previously led to the suspicion that these lobes are especially involved in the storage of associations with shock (Boycott & Young, 1956). However, in many of the experiments reported in earlier papers the situations studied all included crabs, which the octopus were allowed to eat and which were therefore 'positive' figures.

The present series contains decisive evidence that removal of the vertical lobe does not lead simply to indiscriminate attacks on all figures, but only on those that had previously been 'positive'. Thus octopus JEO (Fig. 3) after operation came out to attack crabs, but not to attack a black vertical rectangle. Conversely, JEG (Fig. 4), in which the rectangle had been made first positive and then negative, came out to attack both crabs and the rectangle after operation.

The vertical lobes are thus involved in the setting up and maintaining of visual and tactile associations (Wells & Wells, in preparation). They are not, however, essential for either setting up or maintenance of the memories. In the present series JFL, JDA, JFO and JFA, with less than a quarter of the lobe remaining, all showed some capacity to store information preventing attack on crabs. Performance was, however, very much less accurate than in normal animals. If animals with no vertical lobe remaining may yet store 'negative' memories for short periods this strongly suggests that the appropriate neural configurations can be set up in the optic lobes alone and that the vertical lobes tend to reinforce and maintain them.

SUMMARY

1. Octopuses were fed for some days with fishes in their homes at one end of the tank, and the effect of figures shown at the opposite end was tested.

2. Any small moving object may then be attacked, but the probability of attack is low and the delay long.

3. Crabs and a black horizontal rectangle were attacked more often than other figures. If a figure is withdrawn on the occasions when it is attacked, then the probability of attacks remains low and may slowly decrease.

4. If the octopus is allowed to eat crabs the probability of attacks on them quickly rises towards unity. If food is given when some other figure (say a black vertical rectangle) is attacked, then this figure will later be attacked. An octopus makes attacks more regularly on a figure that has recently been associated with food than on any other figure.

5. This learning to attack a given figure can take place after removal of most of the vertical lobe, but the learning is slower and responses less accurate than in the normal animal.

6. Octopuses from which the vertical lobe has been removed attack crabs more readily than other figures. The system that ensures attack therefore resides elsewhere, probably in the optic lobes.

7. If shocks are given when a previously neutral figure is attacked, then the probability of further attacks on this figure rapidly approaches zero. Attacks on other figures or crabs remain frequent. If the vertical lobe is then removed, no attacks are made on such an originally neutral figure.

8. If a figure that has been associated with food is then rewarded with shocks, the probability of attack falls more slowly than if the figure had been neutral. If the vertical lobe is now removed, the frequency of attacks rises. The memory preventing attack is therefore partly resident in the vertical lobe.

9. The greater the amount of previous training to attack, the more slowly does the animal learn not to attack. This applies also if the training to attack is given after vertical lobe removal.

10. After vertical lobe removal, shocks given when crabs are attacked rapidly reduce the probability of attack if the octopus had not previously eaten crabs while in the laboratory. If such feeding had taken place, the probability of attack falls only slowly without the vertical lobes.

11. The experiments show that information stores associating a given figure with either food or shock can be set up after removal of the vertical lobes but tend to dissipate more rapidly than in a normal octopus.

This work was made possible by the collaboration of the Director and staff of the Stazione Zoologica, to whom I am most grateful. Also to the Nuffield Foundation for financial assistance, to Mr B. B. Boycott for much advice and to Mr R. H. Ansell for histological work.

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THE EFFECTS OF SALINITY CHANGES ON THE RESPIRATORY RATE OF THE PRAWN *PALAEMONETES VARIANS* (LEACH)

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INTRODUCTION

Palaemonetes varians has been classified into distinct varieties based on the size of the egg produced. Variety *macrogenitor*, a fresh-water species, is found in the Mediterranean region but has never been recorded in Britain. The second, variety *microgenitor*, produces a smaller egg and is always found in water containing a certain amount of salt. It has an extensive distribution around the British coasts, being particularly abundant in areas of salt-marsh. Whilst studying the distribution in one such area (Lofts, 1956), it was found that in addition to being widespread in the highly saline marsh-pools, the animal was also present in a system of drainage ditches traversing cultivated land adjacent to the marsh. Here the water was of very low salinity.

The blood concentration of *P. varians* is markedly hypotonic to the medium when in normal sea water (Panikkar, 1939), but this condition changes when the medium is brackish or fresh water. Under these conditions the body fluids remain in a state of definite hypertonicity. Isotonicity is attained when the external medium is equivalent to about 25 g. NaCl per l. (Panikkar, 1941). The average yearly salinity of a marsh-pool is 23·5‰ NaCl, thus the prawns are usually in a state of isotonicity with their medium, being slightly hypertonic in the winter when the pool is diluted by rain, and slightly hypotonic during the hottest months. Those inhabiting the drainage ditches, however, must always be hypertonic to their medium, that is, assuming that both populations of prawns are identical.

The purpose of the present study was to ascertain whether there were any physiological differences between these two groups of animals adapting the one to life in a low salinity environment.

THE HABITAT

The animals used in this investigation inhabited two pools situated within 50 yards of one another and were subjected to the same climatic conditions throughout the year. One population came from a typical salt-marsh pool which lay a little way from high-tide mark. The pool contained highly saline water and was regularly flooded by high tides. The second population inhabited a sluice-pool into which

emptied water from drainage ditches traversing the farm land. This water was slightly brackish and of a very low salinity. The sluice-gates were always kept shut and excess water escaped by overflowing the top. It was never flooded by high tides and a high stone embankment sheltered it from sea-spray.

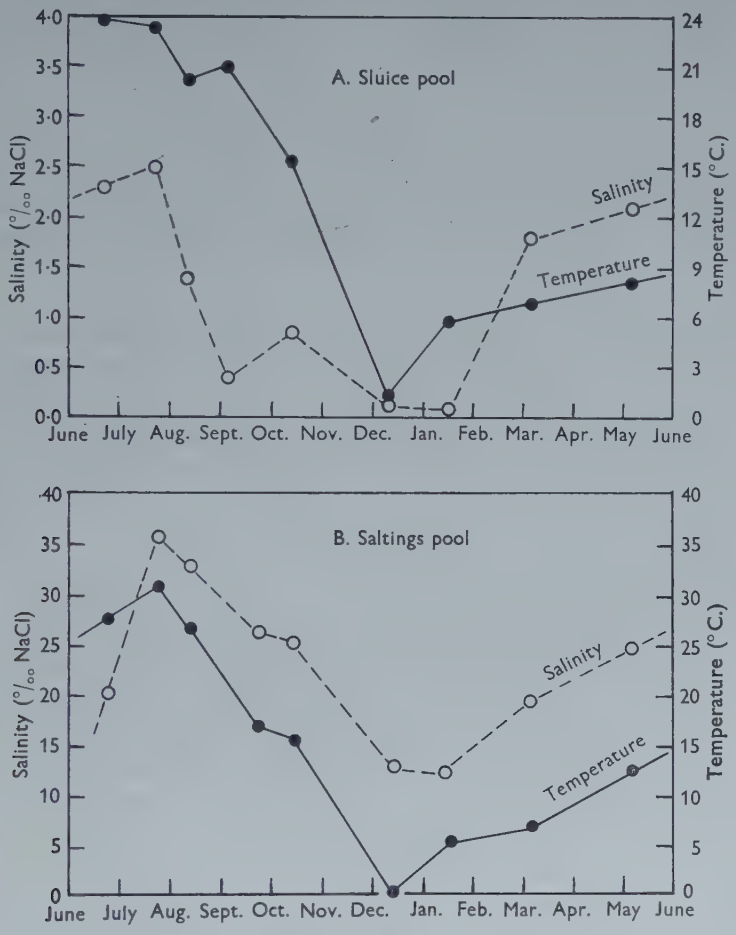

Fig. 1. Seasonal fluctuations in salinity and water temperatures.

Throughout the year both pools undergo gradual seasonal changes in salinity content and water temperature (Fig. 1), and, in addition, the marsh-pool is also subject to sudden changes brought about by entry of sea water at high tides. Changes in salinity closely follow changes in water temperature; during June and July maximum salinities are reached of 36‰ NaCl in the salt-water pool, but only 2·5‰ NaCl in the sluice-pool. The minimum concentration is during January, and figures of 12·8‰ NaCl and 0·1‰ NaCl are recorded for the marsh- and sluice-pools respectively. The average annual salinity is 23·5‰ NaCl in the marsh-pool

and 1·3‰ NaCl in the sluice-pool. These figures show the great difference in the osmotic gradients to which the two populations are subjected.

Both populations are morphologically identical and show no difference in breeding times or in egg size. Egg-bearing females are observed throughout June, and by the end of July both pools swarm with larval prawns. During high tides the larvae in the marsh-pool may become swept out to sea, but the sluice-pool population is not subjected to this dispersal.

MATERIALS AND METHODS

Prawns were transferred to the laboratory and stored in large tanks in a cool room. The two populations were kept in water taken from their respective habitats. No special attempt was made to feed them, but the water was replaced by fresh supplies at monthly intervals, and under these conditions the animals remained in a healthy state for the duration of the investigation.

To study the effects of salinity changes animals were transferred to a medium prepared from clean sea water of the same salinity and pH as the original pond water, and left for a period of 24 hr. to become acclimatized. The rate of respiration was determined and then the salinity of the medium was slightly altered. By a series of dilutions and concentrations effected with tap water and concentrated sea water, the animals were subjected to a range of salinities varying from pure tap water to pure sea water and even concentrated sea water prepared by evaporation and appropriate buffering.

The following uniform procedure was adopted for the measurement of the respiratory rate. Five large bottles were filled to the brim with water of known salinity and immersed in a constant-temperature bath. They were allowed to acquire the temperature of the bath (15° C.). After a 24 hr. acclimatization period in water of the same salinity five prawns of known weight were placed in each of four of the bottles and the fifth was kept as the control without animals. All bottles were then tightly stoppered, care being taken to ensure that no air was trapped inside. Exactly 3 hr. later two samples of water were withdrawn from each bottle and the oxygen content measured.

All salinity estimations were made by titration of a known volume of sample water against standardized silver nitrate solution, with potassium chromate as indicator. This only gave an estimation of the halogens, but since the other salts present were in such minute amounts it was taken as a reading of the total salinity and expressed as grams of NaCl per litre (‰ NaCl).

Oxygen content was estimated by the Winkler (1888) method, which involves the oxidation of manganous hydroxide by the dissolved oxygen in the water to manganic hydroxide. The latter is made to liberate an equivalent of iodine from potassium iodide in an acid medium. The technique used was as follows: (1) to each sample 2 c.c. of Winkler Solution I (33% $MnCl_2$) were run in with a pipette, the point being kept near the bottom of the sample bottle so that a bottom layer was formed; (2) 2 c.c. of Winkler Solution II (30% NaOH + 10% KI) were added in

the same way; (3) the bottle was shaken and the resulting precipitate of manganic hydroxide allowed to settle; (4) 2 c.c. of phosphoric acid were added, and the iodine liberated as a consequence was estimated by titration against standard sodium thiosulphate solution, with starch solution as the indicator. From the results obtained the amount of oxygen in each bottle was calculated, and by subtraction from the control volume the quantity of dissolved oxygen used in unit time by a known weight of animals was estimated.

Before the animals were weighed all adherent water was removed with strips of filter-paper, special care being taken to remove the water in the gill chambers and between the cephalothoracic shield and first abdominal segment. They were weighed in a filter-paper-lined tube and the tube was reweighed after their removal. The difference in the two readings was taken as the weight of the animal.

RESULTS

Both populations of *P. varians* were subjected to salinities ranging from tap water to concentrated sea water of a salinity equivalent to 65‰ NaCl. Both groups proved to be tolerant of a very wide range of salinities, though the limits in each case were slightly different. The salt-water population failed to live for more than 16 hr. in fresh water, but tolerated a salinity as low as 1·7‰ NaCl. for several days. At the other end of the scale salinities of over 60‰ NaCl were tolerated and some

Table 1. *Rate of respiration in* Palaemonetes varians *at different salinities*

Salinity of medium (‰ NaCl)	Respiratory rate (c.c. O_2/gm./hr.)	No. of Readings	S.D. (c.c. O_2/gm./hr.)
	Sluice-pool animals		
0·17	0·74	4	0·013
0·39	0·40	4	0·017
4·59	0·19	4	0·024
9·36	0·19	4	0·001
12·17	0·22	4	—
17·78	0·34	4	0·015
29·52	0·42	4	0·002
36·50	0·56	3	0·022
39·00	0·47	3	0·017
	Marsh-pool animals		
2·9	0·71	3	0·010
4·9	0·72	4	0·001
6·0	0·72	4	0·012
10·3	0·59	4	0·002
12·3	0·54	4	0·223
15·3	0·49	3	0·026
17·4	0·43	3	0·026
22·4	0·39	4	0·016
25·7	0·12	3	0·002
29·0	0·20	4	0·002
34·3	0·26	4	0·002
37·4	0·29	4	0·003
44·0	0·31	4	0·010
52·7	0·37	4	0·002
59·2	0·42	4	0·012

animals remained alive for over a week at a salinity of 66‰ NaCl. Sluice-pool prawns were able to tolerate fresh water, but at salinities above 45‰ NaCl they only survived for a short time.

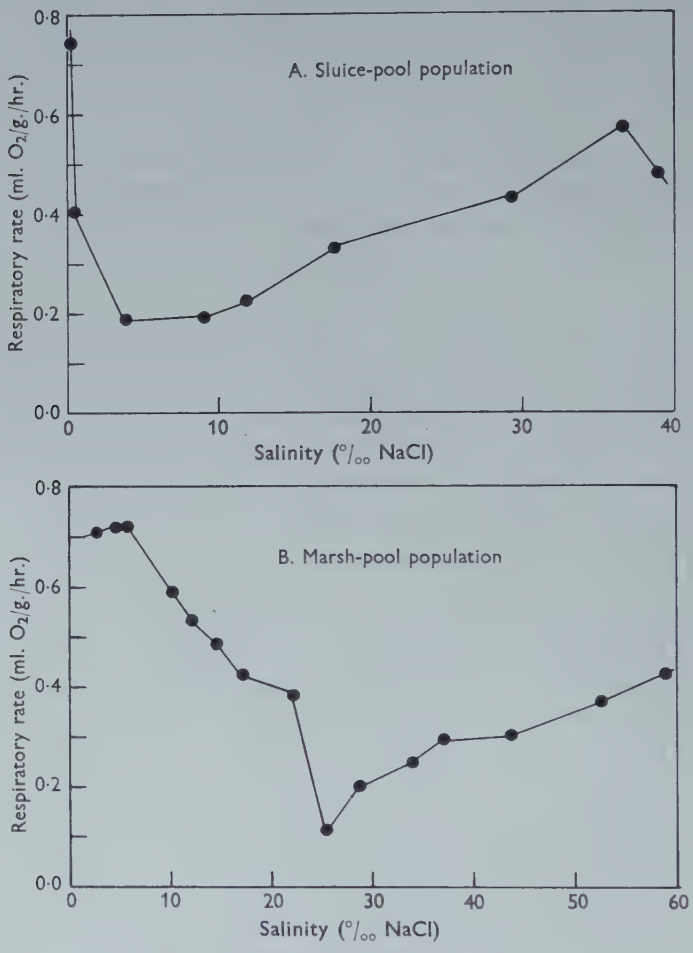

Fig. 2. Respiratory rate of *P. varians* at different salinities of external medium.

The estimated respiratory rate in several different salinities is given in Table 1 and plotted against the salinity of the medium in Fig. 2. In both groups the rate decreases as the salinity increases from tap water. It declines to a minimum rate and then increases again as the external medium becomes more and more concentrated. There is, however, a very marked difference in the salinities at which each group shows the minimal respiratory rate. In the case of the marsh population it was recorded at a salinity of 26‰ NaCl, whereas the sluice-pool animals respired least in a medium of 6‰ NaCl, a difference of 20‰ NaCl.

DISCUSSION

The *P. varians* taken from the marsh-pool have a respiratory rate which is minimal when the animal is isotonic with its medium. In such a state there is no tendency for water to pass across the body wall and energy expended on osmotic work is at its minimum. When the salinity of the medium is changed the animal becomes hypertonic or hypotonic, depending on whether the salinity is decreased or increased, and the respiratory rate increases. This is to be expected, since a change in the osmotic equilibrium necessitates an increase in the osmoregulatory work done by the animal. The energy requirement for osmotic work forms only a small proportion of the total metabolic energy used by an aquatic invertebrate, for example, in the crab *Eriocheir sinensis*, Potts (1954) has calculated that the energy used for osmoregulation is only 0·5 % of the total metabolic energy when the animal is in fresh water. The respiratory rate of both sluice- and marsh-pool populations, however, increases by as much as 600 % between isotonic conditions and extremes in salinity of the external environment. Thus if what is true of *Eriocheir* is also true of *P. varians*, it would seem that these salinity changes not only affect the osmoregulatory mechanism but also some other process which causes a large increase in the total metabolic rate.

The sluice-pool prawns respire least at a salinity of 6‰ NaCl. They are therefore capable of living in low salinity water at a much smaller expenditure of energy than the salt-water population. How this is achieved is a subject for future investigation, but a series of unpublished observations have shown that it is not a lowering of the blood concentration that is responsible. The animals are not isotonic with the medium at 6‰ NaCl. Potts (1954) has demonstrated that a production of urine hypotonic to the blood, even if it is still many times more concentrated than the external medium, can greatly reduce the osmotic work of an animal living in fresh water. Normally *Palaemonetes* produces a urine which is isotonic to the blood, and it would be of great interest to investigate whether this condition differs at all in the sluice-pool prawns. *P. varians* collected from salt-marshes in Kent and Essex have a rate of urine production which is minimal when the external medium is approximately isotonic with the blood, but which increases progressively with increasing dilution of the external medium and also, to a lesser extent, with increasing concentration (Parry, 1955).

This population of prawns inhabiting an almost fresh-water environment probably represents a stage in the physiological adaptation of this species to fresh water, a process which has already been completed in Mediterranean regions. Environmental conditions are far more favourable to the production of a local race in the sluice-pool than in the adjacent marsh-pools. Dispersal of larval prawns by high tides and their intermingling with other populations washed out to sea will minimize the chances of a local race being evolved in the salt-marsh areas, but in the sluice-pool, protected as it is from tidal dispersal of the larvae, offspring that differ from the parents and perhaps more suited to low salinity conditions will be fostered by the more stable conditions, and the evolution of a different race is more likely to occur.

SUMMARY

1. *Palaemonetes varians*, variety *microgenitor*, commonly inhabits only saline water around the British coast. It is, however, abundant in water of extremely low salinity in the Cardiff area, where it occurs in the drainage ditches of farm land adjacent to an area of salt-marsh.

2. Animals from areas of both high and low salinity were subjected, in the laboratory, to a range of salinities varying from pure tap water to concentrated sea water. The rate of respiration at each salinity was measured.

3. The population that inhabited the almost fresh water in the ditches differed physiologically from the salt-water form, although morphologically they were identical.

4. The salt-water population showed a minimum respiratory rate when in a medium of 26‰ NaCl salinity. This was expected, since the animal is isotonic at this salinity and the osmoregulatory work is minimal. The second population respired least when in a medium of 6‰ NaCl. The significance of this is discussed.

This work was carried out in the Department of Zoology, University College, Cardiff, and thanks are due to Prof. James Brough, for providing me with the facilities which enabled me to undertake this investigation and for his help and criticism. I would also like to thank Mr W. A. L. Evans who assisted me in many ways.

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THE EFFECT OF TEMPERATURE UPON THE PERMEABILITY TO WATER OF ARTHROPOD CUTICLES

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INTRODUCTION

Earlier work on the effect of temperature upon the rate of transpiration from arthropods appeared to show a relatively sudden increase in this rate from most insects, spiders and ticks at what has become known as the 'critical' or 'transition temperature'; this change has been ascribed to a correspondingly sudden change in the permeability of a wax layer at, or near to, the surface of the epicuticle (Beament, 1945; Wigglesworth, 1945; Lees, 1947; Davies & Edney, 1952). For the most part measurements have been made in dry air, so that the effect of rising saturation deficit upon the drying power of the air, and the effect of temperature upon the permeability of the cuticle itself, have both contributed to the observed rise in transpiration rate with temperature.

There are two ways of dealing with this effect of increasing saturation deficit; measurements may be made in dry air and the results reduced to rate per unit vapour-pressure difference across the cuticle, or measurements may be made in air whose relative humidity at the different experimental temperatures is so adjusted that the saturation deficit remains constant. Holdgate (1956) and Holdgate & Seal (1956) have recently employed, indirectly, the first method. In the present work the second method has been employed as well; this has not been done with insect material before.

MATERIALS AND METHODS

The greater part of the work was carried out on larvae of *Tenebrio molitor* L., adult *Glossina palpalis* (R.-D.), adult *Calliphora erythrocephala* Mg., and adult *Periplaneta americana* (L.). A few comparative measurements were made on adult *Blatta orientalis* L., the woodlouse, *Oniscus asellus* L., and the centipede, *Lithobius* sp.

The mealworms, blowflies and cockroaches were taken from departmental stocks and had continuous access to food before experiments. The tsetse flies were obtained as pupae from the West African Institute for Trypanosomiasis Research. A small breeding stock was maintained for several months by feeding the flies on rabbit's blood. Woodlice and centipedes were obtained locally and kept until required in large Petri dishes on moist sand together with a few pieces of clean bark and potato.

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The experimental technique used in the present work will be described in some detail, as certain discrepancies that have appeared in earlier work by various authors may be the result of variations in experimental methods. The apparatus used was designed to pass a slow stream of air, at the appropriate temperature and saturation deficit, through an exposure chamber in which the material could be suspended. The value of the saturation deficit chosen was 31·8 mm. Hg, which is the saturation deficit of dry air at 30° C. The use of a stream of air virtually maintains a surface of constant concentration of water vapour at a fixed small distance from the evaporating surface. A steep concentration gradient of water vapour results, and it is reasonable to assume that the rate of evaporation is not affected by the shape of the exposure chamber. However, the effective agent of evaporation over this gradient is diffusion, and the rate at which molecules diffuse is approximately proportional to the square of the absolute temperature, T. This means that irrespective of the value of the saturation deficit the rate of evaporation may be expected to increase with temperature by a factor proportional to T^2. The effect of the increasing rate of diffusion is considerable; the calculated increase in rate at 60° C. as against that at 30° C. is 21 %. Corrections have been applied to all appropriate experimental data for this effect by multiplying by the factor T_1^2/T_2^2, where T_1 was 303° absolute (the lowest experimental temperature of the series) and T_2 was the temperature (degrees absolute) at which the experimental value was obtained.

Dry air was obtained by passing air in a continuous circuit over phosphorus pentoxide and calcium chloride. All other humidities necessary for the provision of the required constant saturation deficit were obtained by bubbling the air stream through solutions of sulphuric acid of appropriate concentrations. A unit of the apparatus using sulphuric acid solution for humidity control is illustrated diagrammatically in Fig. 1; a separate unit was required for each temperature. Air, circulated by a small electric pump, was bubbled through the acid in a large gas jar and thence, via a trap to remove any droplets of acid, to the exposure chamber. The material, suitably mounted, was introduced into the exposure chamber through a wide tube which could be sealed by the constriction of a piece of flexible rubber tubing. The unit providing dry air differed in that phosphorus pentoxide replaced acid at the bottom of the exposure chamber and a long tube of granular, anhydrous calcium chloride replaced the gas jar.

The temperature of the air stream was controlled to within ±0·25° C. by immersing the units of apparatus in a water-bath. Condensation from rising, warm, moist air tended to occur on the inner walls of the glass entry tube above the water-bath surface in the experiments at higher temperatures, when the air stream had a high water content. This condensation was prevented by warming the tube with an electrically heated jacket. The air flow was about 1·5 l./min., a rate equivalent to one complete circulation a minute. Ramsay (1935) has shown that the rate of transpiration from the body surface of the cockroach, unlike that from the tracheal system, is not greatly influenced by variations in the wind velocity; consequently any small variations in wind velocity that occurred within the different units probably caused negligible errors in the results obtained for the rates of transpiration.

Freshly killed animals were used in all experiments. The use of dead material was convenient because support was easier and also because no defaecation occurred during the exposure. It has been shown by Ramsay (1935) for *Periplaneta*, Wigglesworth (1945) for several species of insects and Edney (1951) for several species of woodlice that the rate of loss of water through the cuticle, during short exposures, is effectively the same whether the arthropod is alive or recently dead. Loss of weight was taken as a measure of loss of water, a close approximation in experiments of relatively short duration. Cyanide was used as the killing agent, there being no evidence that it caused any damage to the waterproofing properties of the cuticle as do wax solvents, etc. (Beament, 1945). The spiracles of the insects and centipedes were blocked with cellulose paint to prevent loss of water from the tracheal system.

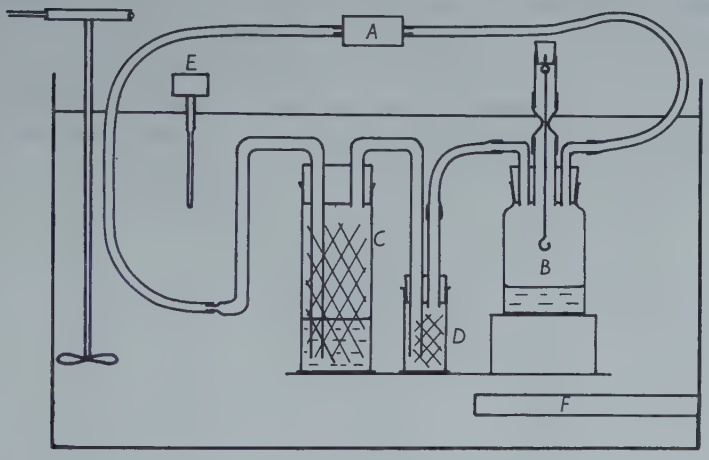

Fig. 1. Diagram of a unit of the apparatus used to provide a stream of air of controlled humidity and temperature. *A*, air pump; *B*, exposure chamber containing sulphuric acid; *C*, humidifier containing sulphuric acid and glass wool; *D*, spray trap containing glass wool; *E*, thermostat; *F*, heater.

In order to compare the rates of transpiration of different species it was convenient to express the losses in terms of surface area, viz. mg. of water/cm.^{2} surface area/hr. The surface areas were obtained from the relationship: surface area $= K \times \text{mass}^{\frac{2}{3}}$, values for K being as follows;

Tenebrio	8·4	Wigglesworth (1945)
Oniscus	13·6	Edney (1951)
Calliphora	10·0	Measured in course of present work.
Lithobius	12·0	Estimated
Glossina	10·0	Estimated
Periplaneta	10·0	Estimated
Blatta	10·0	Estimated

Weighings were made with an aperiodic balance reading to 0·1 mg. The minimum measurable rate of loss in terms of mg./cm.^{2}/hr. depends upon the surface area and the duration of the experiment; in the present experiments at constant saturation deficit it was 0·2 for *Oniscus* and *Lithobius* and 0·1 or less for the insects.

EXPERIMENTAL RESULTS

(*a*) *Preliminary experiments*

Evaporation, from a simple physical system providing a free water surface, into the supposed constant saturation deficit atmospheres was measured. The results, shown in Table 1, indicate that the apparatus provided atmospheres of constant drying power at the six experimental temperatures. The rates of evaporation from the water surface into dry air at a number of temperatures contrast sharply with this constancy, the rate at 60° C. being four times the rate at 30° C.

Table 1. *The rate of evaporation of water, in mg./hr., from a free water surface into a constant saturation deficit of* 31·8 *mm.* Hg, *and into dry air, at a number of temperatures*

Temperature (° C.)	Rate of evaporation	
	Constant saturation deficit	Dry
30	5·3	5·3
35	5·23	—
40	5·11	8·7
45	5·23	—
50	5·38	15·9
55	5·24	—
60	—	22·8

Whilst it is probable that at any one temperature the same physico-chemical systems control the movement of water through and from the cuticle in all normal individuals of a species, it is likely that there will be a certain individual variation in the absolute permeability of the cuticle within the species. If this is the case, and the effect of temperature alone upon these physico-chemical systems, as far as waterproofing is concerned, is not to be obscured by individual variations in the levels of water loss, it is preferable to expose each individual to the full range of experimental temperatures rather than use a different individual at each temperature. A difficulty that has frequently been referred to is a progressive decline in the rate of transpiration from a specimen in constant conditions. This was the case with *Periplaneta* (Ramsay, 1935), *Agriotes* larvae (Wigglesworth, 1945) and woodlice (Bursell, 1955). Measurements were therefore made of the consistency in rate of transpiration from various arthropods when exposed to one temperature and saturation deficit until desiccation was well advanced. In *Locusta migratoria*, the rate of transpiration into dry air at 30° C. had decreased to less than a quarter of the initial rate after 14 hr., although the water content was reduced by less than 20%. The

fall in rate was rapid at first but after a few hours much more gradual. This asymptotic type of decrease was not found in *Glossina palpalis* or *Calliphora erythrocephala*, where the rate was more nearly constant. By the time the water content had been reduced to 50% the rate was only about 10% below the initial rate. As a result of these measurements locusts were regarded as unsuitable material for the purposes of the present studies.

It is clearly necessary to design the experiments in order to keep the total loss of water from the material during the experiment to a minimum, and thereby prevent any marked decline in transpiration rate associated with increasing desiccation during the later exposures. The use of a fixed saturation deficit at all experimental temperatures is valuable in that the very high rates of water loss (and attendant more rapid desiccation) associated with transpiration into dry air at high temperatures are eliminated. In the experiments at constant saturation deficit described below, total water loss was very much less than 50%, so that with the insect material it was hoped that transpiration was little affected by desiccation. Woodlice and centipedes have cuticles considerably more permeable than those of terrestrial insects, and at higher saturation deficits the rate of transpiration was found to fall off rapidly with time, and special allowance was made for this in the design of some of the experiments with these animals (see § (*b*) (1) below).

(*b*) *The relation between rate of transpiration and temperature*

(1) *In* Oniscus asellus. When freshly killed woodlice were exposed for $\frac{1}{4}$ hr. periods to the constant saturation deficit atmospheres of 31·8 mm. Hg at six temperatures in the order 30, 40, 50, 55, 45, 35 and 30° C., it was found that there was a small rise in the rate of transpiration during the first three exposures, but then the rates decreased and the results for the intermediate temperatures did not fall on the same line as the first three. As it was not practicable to reduce the period of exposure, each individual was exposed at only three temperatures, either 30, 40 and 50° C. or 35, 45 and 55° C. The order of exposure to these temperatures was varied so that some individuals were exposed, first, to the highest temperature, some to the intermediate one and some to the lowest. This served to compensate for any falling off in rate caused by desiccation as the experiment progressed. The results are shown in Table 2 and are plotted against temperature in Fig. 2. There are two curves on Fig. 2 relating to *Oniscus*, one for each 'temperature group'. Both are approximately straight and rise only slightly, which indicates a very small increase in permeability with temperature. Edney (1951) reports a similar result for *Porcellio scaber* Latr. at constant saturation deficit.

(2) *In* Lithobius *sp.* Measurements were made of the rate of transpiration, at three temperatures and a saturation deficit of 31·8 mm. Hg, from freshly killed centipedes with all spiracles occluded. The order of exposure was varied as with *Oniscus*. The results are given in Table 2 and plotted against temperature in Fig. 2. There is an increase in rate with temperature, but not nearly so great an increase as that found in insects. The individual variation in rate was considerable, as

Table 2. *The rate of transpiration, in mg./cm.²/hr., from certain arthropods into a slow stream of dry air, and into air at a constant saturation deficit of* 31·8 *mm.* Hg. *Each entry is a mean and is followed by the standard error*

Material and Temperature (° C.)	Constant saturation deficit (31·8 mm. Hg)			Dry air		
	Mean rate and S.E.	Mean rate corrected for diffusion	$\log \frac{R \times 10^6}{pT^2}$	Mean rate and S.E.	Mean rate corrected for diffusion	$\log \frac{R \times 10^6}{pT^2}$
Calliphora	(Mean of 9)			(Mean of 3)		
30	1·62 (0·15)	1·62	$\bar{1}$·74	1·2 (0·09)	1·2	$\bar{1}$·61
35	2·07 (0·14)	2·00	$\bar{1}$·84	2·1 (0·17)	2·0	$\bar{1}$·72
40	2·54 (0·20)	2·38	$\bar{1}$·91	3·0 (0·38)	2·8	$\bar{1}$·74
45	2·90 (0·16)	2·63	$\bar{1}$·96	5·2 (0·72)	4·7	$\bar{1}$·85
50	4·05 (0·25)	3·56	0·09	9·4 (0·72)	8·3	$\bar{1}$·99
55	4·75 (0·27)	4·05	0·14	15·5 (1·13)	13·2	0·09
Glossina	(Mean of 9)			(Mean of 3)		
30	0·40 (0·04)	0·40	$\bar{1}$·14	0·53 (0·02)	0·53	$\bar{1}$·26
35	0·67 (0·06)	0·65	$\bar{1}$·36	0·90 (0·04)	0·87	$\bar{1}$·35
40	0·96 (0·08)	0·90	$\bar{1}$·49	1·35 (0·13)	1·26	$\bar{1}$·40
45	1·30 (0·08)	1·18	$\bar{1}$·61	2·00 (0·00)	1·82	$\bar{1}$·44
50	1·82 (0·10)	1·60	$\bar{1}$·74	3·00 (0·21)	2·64	$\bar{1}$·49
55	2·08 (0·12)	1·77	$\bar{1}$·78	— —	—	—
60	— —	—	—	7·00 (0·85)	5·82	$\bar{1}$·63
Tenebrio	(Mean of 23)			(Mean of 3)		
30	0·18 (0·01)	0·18	$\bar{2}$·79	0·24 (0·01)	0·24	$\bar{2}$·91
35	0·20 (0·02)	0·19	$\bar{2}$·82	0·24 (0·01)	0·23	$\bar{2}$·78
40	0·25 (0·02)	0·23	$\bar{2}$·90	0·33 (0·01)	0·31	$\bar{2}$·78
45	0·38 (0·03)	0·35	$\bar{1}$·07	0·61 (0·03)	0·55	$\bar{2}$·92
50	0·99 (0·14)	0·87	$\bar{1}$·47	1·39 (0·09)	1·22	$\bar{1}$·16
55	1·90 (0·21)	1·62	$\bar{1}$·74	2·81 (0·33)	2·40	$\bar{1}$·35
60	— —	—	—	6·26 (0·87)	5·19	$\bar{1}$·58
Periplaneta	(Mean of 6)			(Mean of 6)		
20	— —	—	—	0·47 (0·03)	0·49	$\bar{1}$·49
30	1·56 (0·23)	1·56	$\bar{1}$·73	1·72 (0·09)	1·72	1·77
35	3·09 (0·31)	2·99	0·01	4·13 (0·34)	3·99	0·01
40	4·49 (0·29)	4·21	0·16	6·75 (0·21)	6·32	0·10
45	5·18 (0·33)	4·70	0·21	11·15 (0·53)	10·12	0·19
50	7·21 (0·40)	6·34	0·34	19·04 (0·65)	16·76	0·30
55	5·97 (0·50)	5·09	0·24	24·52 (0·63)	20·92	0·29
Blatta	(Mean of 3)					
30	1·54 (0·16)	1·54	$\bar{1}$·72			
35	2·85 (0·15)	2·76	$\bar{1}$·98			
40	4·07 (0·42)	3·81	0·12			
45	6·35 (0·21)	5·77	0·30			
50	8·04 (0·05)	7·08	0·38			
55	6·35 (0·26)	5·42	0·27			
Oniscus	(Mean of 6)					
30	3·69 (0·17)	3·69	0·10			
40	4·28 (0·16)	4·01	0·14			
50	5·00 (0·12)	4·40	0·18			
35	4·19 (0·19)	4·05	0·14			
45	4·63 (0·21)	4·20	0·16			
55	4·96 (0·20)	4·23	0·16			
Lithobius	(Mean of 12)			(Mean of 5)		
20	— —	—	—	3·7 (0·36)	3·9	0·39
25	— —	—	—	5·0 (0·40)	5·2	0·37
30	8·5 (0·67)	8·5	0·46	6·8 (0·40)	6·8	0·37
35	— —	—	—	9·5 (0·57)	9·2	0·38
40	11·2 (0·87)	10·5	0·56	12·4 (0·58)	11·6	0·36
45	— —	—	—	15·0 (0·59)	13·6	0·31
50	13·7 (1·05)	12·1	0·62	16·6 (0·81)	14·6	0·24

shown by the high standard errors, but the trend was consistent with all twelve individuals. A statistical analysis showed that the difference in rates at 30 and 40° C. and at 40 and 50° C. was significant. ($P < 0{\cdot}001$ and $< 0{\cdot}01 > 0{\cdot}001$ respectively.)

Results were also obtained for the rate of transpiration from each of five individuals into dry air at 5° C. intervals from 20 to 50° C. They are included in Table 2 and Fig. 3. In this particular experiment the values for the higher temperatures were probably too low owing to a decreased 'availability' of water as desiccation proceeded, otherwise the curve is close to a saturation deficit/temperature curve.

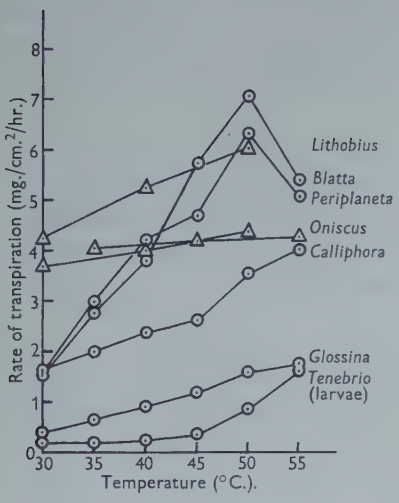

Fig. 2

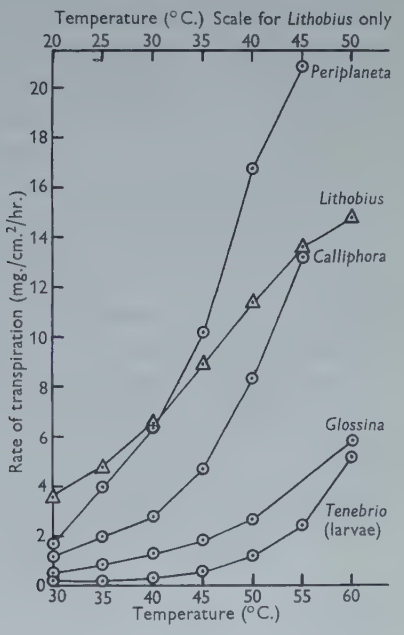

Fig. 3

Fig. 2. The rate of transpiration from certain arthropods into a constant saturation deficit of 31·8 mm. Hg at various temperatures. *Lithobius* plotted as rate $\times \frac{1}{2}$.

Fig. 3. The rate of transpiration from certain arthropods into dry air at various temperatures.

(3) *In* Tenebrio molitor *larvae*. The rate of transpiration was measured from recently dead *Tenebrio* larvae, with occluded spiracles, when exposed to a saturation deficit of 31·8 mm. Hg at six temperatures, and to dry air at seven temperatures. The results are included in Table 2 and Figs. 2 and 3. The curve obtained from measurements at constant saturation deficit and shown in Fig. 2 indicates a gradual, but progressive increase in permeability of the cuticle over the temperature range studied, 30–55° C.

(4) *In* Glossina palpalis *and* Calliphora erythrocephala. The rates of transpiration from dead *Glossina* and *Calliphora*, with thoracic spiracles occluded, into dry air and air at constant saturation deficit, are given in Table 2. They are shown plotted against temperature in Figs. 2 and 3. It will be seen that *Glossina* cuticle is relatively

less permeable than that of *Calliphora*. The graph of rate of transpiration, measured at constant saturation deficit, against temperature (Fig. 2) for *Glossina* is sloping and approximately linear, whilst that for *Calliphora* is sloping but slightly curved; the apparent cusp at 45° C. is not statistically significant. The cuticles of both species are thus shown to become progressively more permeable as the temperature rises from 30 to 55° C.

(5) *In* Periplaneta americana *and* Blatta orientalis. Measurements were made of the rate of transpiration, into air at constant saturation deficit and at six temperatures, from each of six individuals of *Periplaneta* and three of *Blatta* when freshly killed and with spiracles blocked. Six other *Periplaneta* were exposed to dry air at a range of temperatures from 20 to 55° C. The results are included in Table 2 and shown graphed on Figs. 2 and 3. The two species show very similar permeability changes; it is important to notice the marked and progressive increase in permeability over the range 30–50° C. Both species show an apparent decrease in permeability at 55° C.; whilst this could be the result of increasing desiccation, it may result from some hygroscopic effect at the high relative humidity (73 %) necessary at 55° C. in the constant saturation deficit experiments.

Table 3. *The rate of transpiration, in mg./cm.²/hr., from adult* Periplaneta americana, *into a slow stream of air at a constant saturation deficit of* 31·8 *mm.* Hg *before, and after, treatment with chloroform to remove the cuticular wax. Each entry is the mean of six measurements and is followed by the standard error*

Temperature (° C.)	Before de-waxing			After de-waxing		
	Mean rate and S.E.	Mean rate corrected for diffusion	$\log \frac{R \times 10^6}{pT^2}$	Mean rate and S.E.	Mean rate corrected for diffusion	$\log \frac{R \times 10^6}{pT^2}$
30	1·42 (0·16)	1·42	$\bar{1}$·69	14·29 (0·95)	14·29	0·69
40	4·49 (0·46)	4·21	0·16	14·24 (1·55)	13·34	0·66
50	7·68 (0·98)	6·76	0·32	15·31 (1·45)	13·47	0·67

An experiment was performed to investigate whether this progressive increase in permeability of insect cuticle, with increase of temperature, is entirely dependent upon the presence of the wax of the epicuticle. This is difficult to prove by direct experiment with the majority of insects, as the wax is normally protected by an outer layer of cement that is not removable without drastic treatment, such as boiling in chloroform. In the cockroach, however, the wax is freely exposed on the surface of the cuticle, the cement layer being embedded in wax rather than being entirely superficial (Kramer & Wigglesworth, 1950; Dennell & Malek, 1955). This allows much of the soft waxy material of the epicuticle to be removed readily by brief washing in cold chloroform. The permeability of the de-waxed cuticle may then be investigated in the usual manner.

Six freshly killed individuals of *P. americana* were used, each with spiracles and body openings occluded. The rate of transpiration from each was measured during

$\frac{1}{4}$ hr. exposures to the constant saturation deficit of 31·8 mm. Hg at 30, 40 and 50° C. The specimens were then washed in cold chloroform for 3–4 min., and transferred to a moist atmosphere for $\frac{1}{2}$ hr. to allow the adherent chloroform to evaporate. When they were free from chloroform odour their weight had decreased by about 100 mg. (*c.* 1 % of the original body weight) owing to some evaporation of water. They were then re-exposed at the same temperatures and saturation deficit as used before de-waxing.

The results are summarized in Table 3. The permeability of the cuticle increases fivefold between 30 and 50° C. before de-waxing, but is unaffected by temperature afterwards. All the progressive change in cuticle permeability is thus dependent upon the presence of cuticular wax. It will be seen from the data in Tables 2 and 3 that the cockroach cuticle without its soft wax covering has a permeability even greater than that of the cuticles of the woodlice and centipedes.

(*c*) *The relation between the rate of transpiration and saturation deficit at high temperatures*

If the limiting factor for the rate of transpiration from an insect is the drying power of the air, it is to be expected that the rate of transpiration per unit saturation deficit will be constant at one temperature, whatever the value of the saturation deficit used during the measurement. Comparison of the rates measured in a saturation deficit of 31·8 mm. Hg and in dry air indicate that, in *Tenebrio* larvae and *Glossina*, the rate is relatively greater when determined at the lower saturation deficit. This may be caused by desiccation, the effects of which will normally appear sooner in experiments carried out with dry air than in those using moist air, but may also indicate that some other factor limits the rate of evasion at high saturation deficits.

The difference is much less apparent in the results obtained for *Calliphora*. Measurements were made of the rates of transpiration from each of eighteen individuals of *Calliphora* into saturation deficits of 31·8, 65·8 and 118·0 mm. Hg at 55° C. The ratios of loss per unit saturation deficit were 1·0:1·0:0·93, which is reasonably close to a direct proportionality between rate of transpiration and saturation deficit at this one temperature.

DISCUSSION

We may conclude that the cuticle permeability of *Oniscus* and *Lithobius* is little affected by temperature, over the range studied, as their rates of transpiration at constant saturation deficit do not increase greatly. This agrees with the conclusions of Edney (1951) and Cloudsley-Thompson (1950). The absolute permeability of the centipede and woodlouse cuticle is high compared with that of most terrestrial insects that have been studied. These two characteristics, high absolute permeability and low increase of permeability with temperature, are expected with species which apparently lack a continuous wax layer to the cuticle.

The present data for insects show certain irregularities that may derive from some unaccounted experimental hazard. For example, the measured rate of transpiration from *Calliphora* at constant saturation deficit and 45° C. is too low to allow the construction of an otherwise smooth curve when the rate of transpiration/temperature relationship is drawn. Similarly, the rates at 55° C. and 31·8 mm. Hg saturation deficit for *Blatta* and *Periplaneta* are exceptionally low. We must recognize that certain experimental errors arise with the usual methods for the determination of cuticle permeability and should not place too great a reliance upon small apparent changes in permeability from temperature to temperature; marked trends, however, are valid and may allow certain definite conclusions to be drawn.

The data obtained for the rates of transpiration into dry air at various temperatures from insects (Wigglesworth, 1945), ticks (Lees, 1947) and spiders (Davies & Edney, 1952) led to the conclusion that at a fairly definite temperature, dependent upon the species, the cuticle permeability greatly increased. This increase was believed to result from some transition in a discrete wax layer of the epicuticle. When Beament (1945) extracted this wax and deposited it upon suitable membranes he found that the permeability of these membranes showed temperature-dependent changes generally similar to those of the intact insect. He regarded the basis of these permeability changes to be a single change in the wax, probably a 'crystalline transition' of the type Müller (1932) found to occur in certain long-chain normal paraffins (C_{24}–C_{44}) a few degrees below their melting-points. It is possible to envisage that such a transition, producing an abrupt change in the dimensions and spacings of the wax molecules, in a suitably orientated wax layer could lead to a sharp increase in permeability to water of the system. Such a single change could explain the existence of a certain degree of permeability below the transition temperature and a higher one above it, but not a continuous rise in permeability above this temperature. The measurements made at constant saturation deficit clearly indicate that there is a real, continuous change with temperature in the permeability of the cuticle. This was so over the whole experimental range of 30–55° C. for *Glossina* and *Calliphora*, from 30 to 50° C. for *Blatta* and *Periplaneta*, and from at least 40 to 55° C. for *Tenebrio* larvae. The use of dry air at all temperatures, with its necessarily increasing drying power, has generally tended to obscure the existence of this continuous change which produces a real, progressive increase in cuticle permeability with temperature.

A single transition of the Müllerian type cannot explain continuous permeability changes. Chibnall, Piper, Pollard, Williams & Sahai (1934) have shown that the cuticular waxes of insects are complex mixtures of long-chain paraffins, primary alcohols, fatty acids, etc. Beament (1955) found that shorter-chain, volatile paraffins, alcohols, etc., are additionally present in the soft wax of *Periplaneta*. Such complex mixtures of waxy materials will not possess sharply defined physical characteristics. Recently, Holdgate & Seal (1956) have found that no 'crystalline transition' is demonstrable in the chloroform-extracted, primary cuticular wax of *Tenebrio* pupae. This primary wax showed a very diffuse melting-point with parts

melting from about 35 to 55° C., although most melted by 42° C. It is possible that physical changes in the cuticular waxes, which have such complex chemical composition and variable spatial distribution within the epicuticle, could produce a continuous change in permeability over a fairly wide temperature range.

Holdgate & Seal (1956) believe that the cuticle permeability changes are basically exponentially related to the temperature, as is the movement of water vapour and certain gases by activated diffusion through some organic membranes (Barrer, 1939; Doty, Aiken, & Hermann, 1944). To test this hypothesis with the present results values of $\log_{10}\frac{R}{pT^2}\times 10^6$ were calculated, and these values are included in Table 2. (R is the rate of transpiration, in mg./cm.^{2}/hr., measured at a saturation deficit of p mm. Hg and an absolute temperature of T°.) The values of $\frac{R}{pT^2}$ represent, on an arbitrary scale, the rate of transpiration per unit saturation deficit, with correction for the increasing rate of diffusion with rising temperature. These values therefore give an indication of the permeabilities of the cuticles concerned. If the cuticle permeability is strictly exponentially dependent upon temperature the graphs of log permeability against temperature, or the reciprocal of temperature should be linear. Fig. 4 shows the graphs of log 'permeability' $\left(\log_{10}\frac{R}{pT^2}\right)$ against $1/T$ (using the data obtained with the insect material at constant saturation deficit). The graphs approximate to linearity, with the exception of that for *Tenebrio* larvae, which is decidedly curved. (It is unlikely that this difference in shape can be correlated with the presence of an outer cement layer in the cuticle of *Tenebrio*, and with its absence, as such a layer, in the other species used, since Holdgate & Seal obtained similar curved graphs for freshly moulted *Tenebrio* pupae and *Rhodnius* nymphs which both lack a cement layer at that stage.) The linearity of the majority of the plots of log 'permeability' against $1/T$ may indicate that the cuticle permeability of several species of insects is exponentially dependent upon temperature, and may obey the relationship $P=P_0e^{-E/T}$, where P is the permeability constant and P_0 and E are constants; this is a relationship characteristic of transmission by activated diffusion (see Barrer, 1939).

It seems that at the present time there are still too many unknown factors to enable us to come to any final conclusions as regards the physical basis of the temperature-dependent changes in the permeability of insect cuticle. There is a certain inconsistency in results obtained by different workers, which might be overcome by refinement and standardization of technique; but even in the hands of one worker there are interspecific differences of such a nature as to indicate either a very considerable experimental error or a complex interaction of factors determining the effect of temperature upon cuticle permeability. Thus the curves for log permeability against temperature are sometimes approximately linear, sometimes curved upwards or downwards. Broadly speaking, there appear to be two possible mechanisms that might lead to an approximately exponential relationship between temperature and permeability: activated diffusion through a cuticle

that does not itself undergo physical change, and progressive change towards greater permeability of a complex wax layer in the cuticle. Between these two mechanisms, or various combinations of the two, it is impossible to distinguish using the present techniques, and a new method of approach is required.

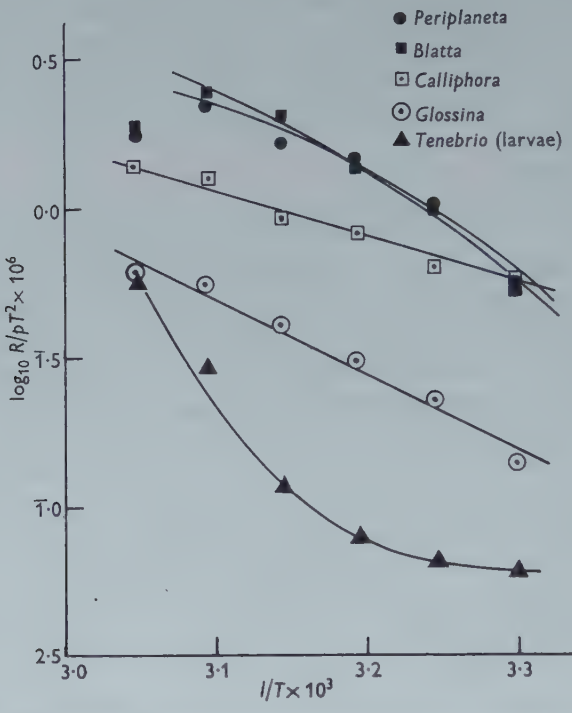

Fig. 4. Graphs of $\log_{10}(R/pT^2) \times 10^6$ against $1/T$ for a number of insects. Derived from measurements of rate of transpiration into a constant saturation deficit of 31·8 mm. Hg.

SUMMARY

1. The effect of temperature alone upon the permeability of arthropod cuticles has been investigated by measuring the rate of transpiration into a constant saturation deficit at a number of temperatures.

2. The permeability of the cuticles of *Oniscus asellus* and *Lithobius* sp. is little affected by temperature.

3. The cuticles of *Calliphora erythrocephala*, *Glossina palpalis*, *Blatta orientalis* and *Periplaneta americana* become progressively more permeable as the temperature is raised from 30 to 50, or 55° C. That of *Tenebrio molitor* larvae behaves similarly, but the increase in permeability is less marked until the temperature rises above 40° C.

4. The progressive increase in permeability of the cuticle of *Periplaneta* is entirely eliminated if the soft wax coating is removed.

5. The increase in permeability of the insect cuticle with temperature is approximately exponential and cannot result from a single, abrupt change in a wax layer at a 'critical temperature'. Possible physical bases for a continuous change are discussed.

I wish to thank Prof. E. B. Edney for his very valuable advice, discussion and encouragement during the course of this work. I gratefully acknowledge the receipt of a Birmingham University Research Scholarship held during this period.

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AN INEXPENSIVE TYPE OF SOUND-PROOF ROOM SUITABLE FOR ZOOLOGICAL RESEARCH

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(*Received 22 June* 1956)

INTRODUCTION

Modern developments in electronic technique have now rendered possible and attractive the electrophysiological study of auditory organs and the critical analysis of the sounds produced by animals. But in order to undertake such experimental work the provision of some form of sound screening or insulation is often required. The standard type of sound-proof room giving virtually full insulation at all audio-frequencies is of course ideal and for some types of work essential. Such rooms are, however, very expensive to construct, since, in order to be impervious to the lower frequencies in the auditory spectrum, there must be at least two constructional shells sufficiently massive to be themselves non-resonant and to support between them the considerable weight of sand which is necessary for insulation. Moreover, while a completely an-echoic room will probably be an unnecessary or unattainable refinement, most types of experiment will necessitate that the internal walls of the room have a high coefficient of sound absorption (i.e. be more or less non-reflective) over a wide range of audio-frequencies. This will require, in addition, a lining of glue-foam, 'fibre-glass' board or other similar material. Specifications for the construction of sound-proof rooms of the standard type will be found in the book by Constable & Constable (1949) and in the publications of the Acoustical Materials Association.

Fortunately, complete sound-proofing over the full range is not necessary for many types of zoological work. It may be sufficient to eliminate the higher frequencies only, provided the others are reduced by a known amount. This is particularly true of work with insects, and with the songs and call notes of the passerine birds, and with the sounds produced by the smaller mammals. The equipment here described was developed at the Madingley Ornithological Field Station of the Cambridge University Department of Zoology for the investigation and analysis of the innate and learned components of the sounds produced by passerine birds (Thorpe, 1954, 1955 and 1956). For such experiments it was necessary to be able to isolate birds, from the earliest nestling stages onwards, from the songs and call notes of the same and related species in sound-proof rooms with a low degree of echo. For this purpose sounds below 2 kc./sec. could be disregarded, and it was possible to adopt a mode of construction very much cheaper than the standard type. The details are published now in the hope that they may be of interest to biologists working in a number of different fields.

CONSTRUCTION

The essence of the constructional plan consists in the use of *Thermacoust* (wood-wool slabs) as the main building material. This has the great advantage that it is itself sufficiently strong and rigid to constitute the actual framework of building and yet, when smoothly plastered on the outer surface, it confers a high degree of sound insulation (from the outside), while the unplastered inner surface is reasonably absorptive of sound. It is thus possible to avoid the expense of building the two massive and independent double frameworks necessary to contain the two separate layers of insulating material (sand, broken cork, dried eel-grass, etc.) required for the standard type of sound-proof room. All that is required is to construct two independent shells of Thermacoust, plastered on the outsides, each supported by a separate light wooden framework.

The present paper is based on the experience gained in building two sound-proof rooms. The description given below refers primarily to the second (room A) which incorporates lessons learned in the building of the earlier one (room B). Although, as will be seen from the tables, room B actually produces a slightly better sound attenuation than does room A, this is because room B was at first unsatisfactory in certain respects and was subsequently modified at considerable extra cost. In view of this elaboration it must be regarded as the less efficient of the two. The design of room A, if properly carried out, is both efficient and fully adequate for its purpose. The construction of room A is illustrated in Figs. 1–3. The principal features are as follows:

Floor. The room is built on the wooden floor of a pre-existing hut, the foundations of which consist of four dwarf brick walls on concrete footings, running lengthwise of the hut. The floor of the room consists of a bitumen-bonded fibre-glass mat lying on fibre board which in its turn lies on the floor of the hut. The area of the bitumen-bonded fibre-glass mat is slightly greater than the plan area of the outer shell, so that it projects slightly round the edges.

Walls and roof. There are two shells, each consisting of 2 in. Thermacoust supported on a 2 × 1 in. wooden framework. This framework lies in the 3 in. space between the two Thermacoust shells—so that there is a 1 in. space between the battens on the inside of the outer shell and those on the outside of the inner shell. The nails holding the Thermacoust to the wood penetrate only a short distance into the wood. The outside of each shell is covered in a fairly thick layer of plaster.

Doors. The doors open outwards in order to give the maximum of useful space inside; the outer door is therefore somewhat larger than the inner. All edges of the doors are bevelled and shut against sheet sponge rubber so that there is a tight seal all round (Fig. 2A).

Since the Thermacoust sheets were too narrow for the outer door to be covered by one sheet, special precautions to prevent the plaster cracking over the joint were necessary. The door was therefore covered with a thin metal sheet mounted over fibre glass; this gives increased rigidity without a great increase in weight or clumsiness.

Windows. Each shell has a double window—one sheet of Perspex and one of glass (Fig. 2B). Each sheet is supported in sponge rubber held in a wooden frame, the latter being attached to the side of the Thermacoust (inside the Thermacoust in the inner shell, and outside in the outer).

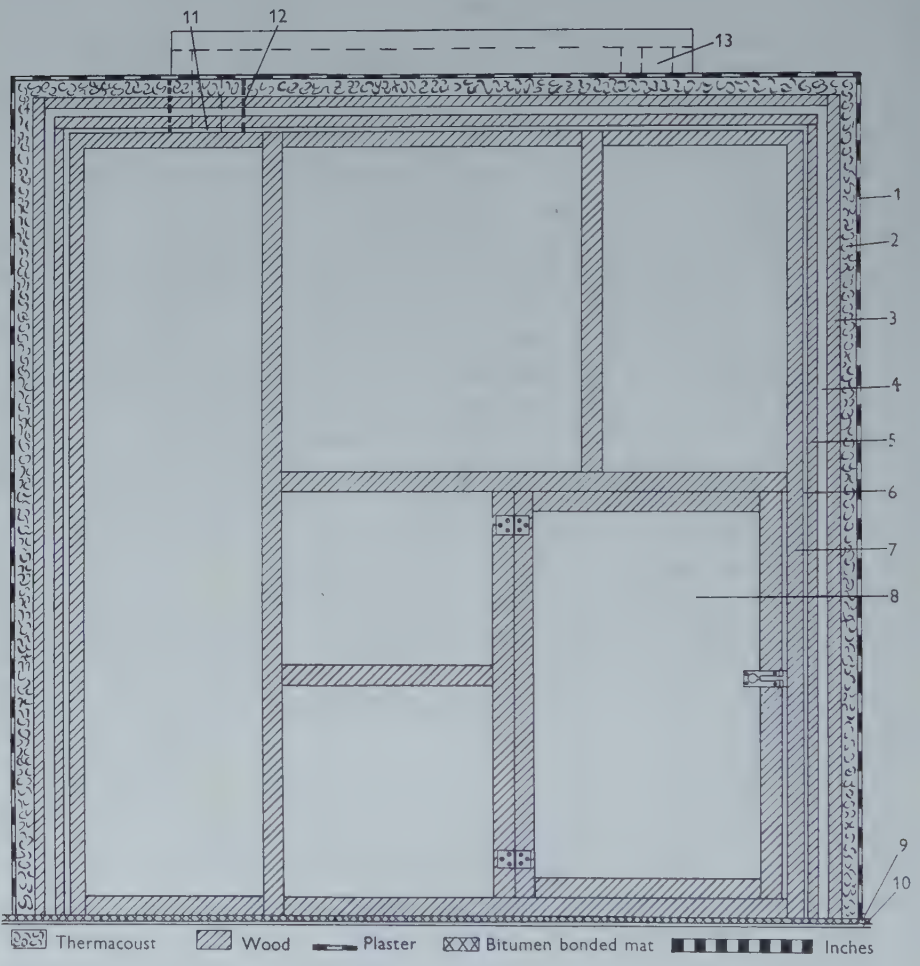

Fig. 1. Front elevation with front of outer shell removed.

Explanation of Figs. 1–3

1, plaster skin of outer shell; 2, Thermacoust of outer shell; 3, wood frame of outer shell; 4, space between inner and outer shells; 5, wood frame of inner shell; 6, plaster face of inner shell; 7, face of wooden frame of inner shell; 8, door; 9, bitumen-bonded mat; 10, hardboard sheet; 11, ventilator shaft; 12, packing to ventilator shaft; 13, open end of ventilator shaft.

Door: 14, wooden beading frame; 15, galvanized iron sheet; 16, fibre glass; 17, plaster; 18, Thermacoust; 19, rubber facing to door frame.

Window: 20, Perspex; 21, glass; 22, rubber seating; 23, wood block; 24, Thermacoust; 25, plaster skin.

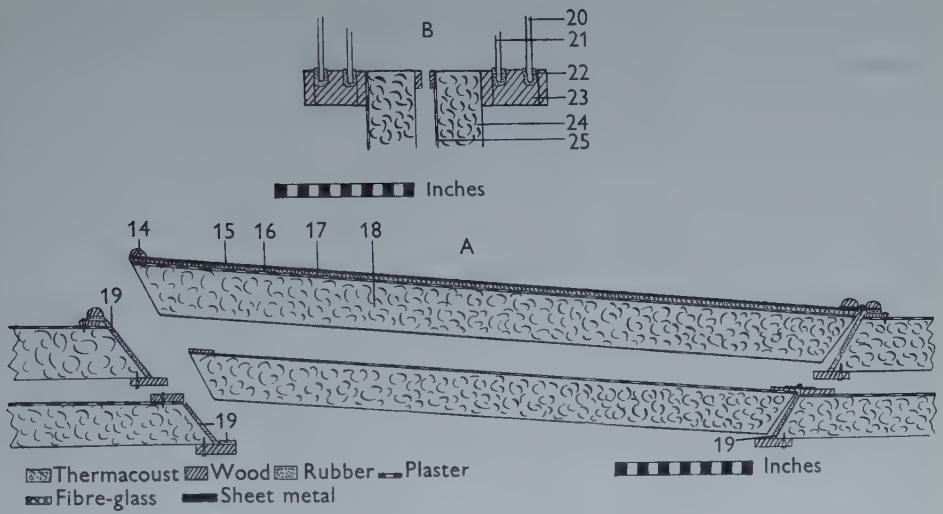

Fig. 2. Construction of A door and B window.

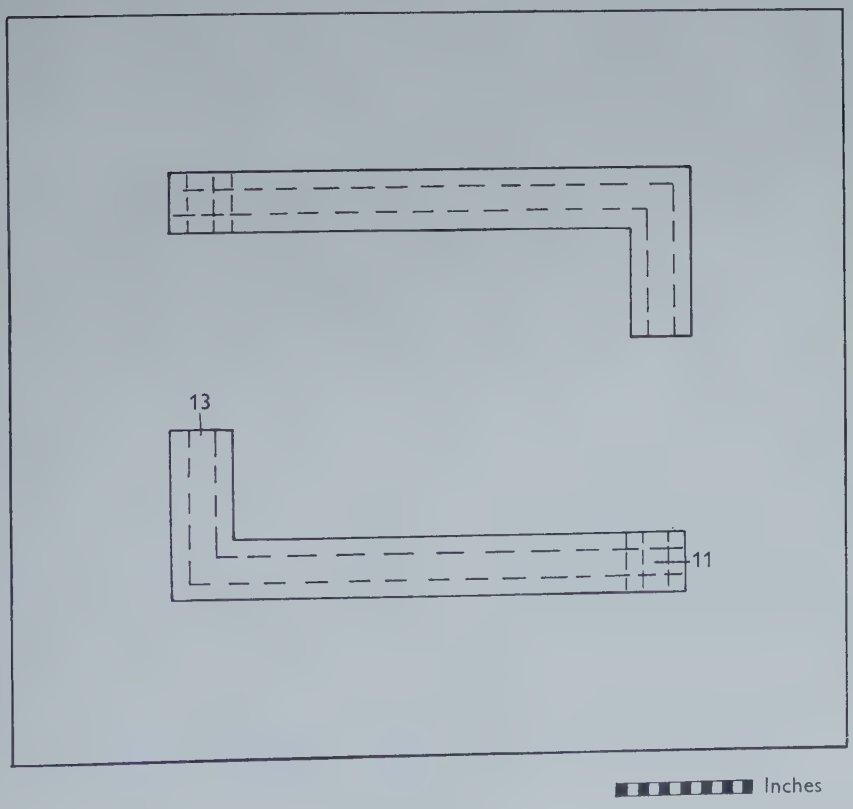

Fig. 3. Roof plan showing ventilation.

Ventilators. The room is ventilated by two Thermacoust ducts, the space inside being 3 in. square. Each duct has two right-angle bends, one in the plane of the roof, the other at right angles to this, leading down into the interior. These ventilator shafts thus form the only points of contact between inner and outer shells above floor level; where the shafts penetrate the shells they are insulated from them by a packing of sponge rubber. The outer surface is plastered (Fig. 3).

Room B. This differed from room A primarily in that the inner shell was constructed of *two* layers of Thermacoust mounted on either side of a common wooden frame, and in having the floor similar to the inner shell.

EFFICIENCY

The performance of the rooms was tested by generating tones of known frequency and amplitude immediately outside and testing their intensity inside by means of a Ribbon Microphone and a Dawe Sound Level Meter (type No. 1400c). In order to overcome the position errors produced by standing waves, the sound was produced by means of a Warble Tone Generator at 110 db.

Table 1. *Showing efficiency of sound-proof rooms A and B expressed as attenuation in decibels of a sound of* 110 *db. produced by a Warble Tone Generator at different average frequencies (expressed in cycles per second)*

The internal ambient noise level due to the presence of the observer, etc., was 38 ± 1 db. This was increased to 54 ± 1 db. by fluorescent lighting. Figures for sound level are taken relative to the human auditory threshold reckoned as 0·0002 dynes/cm.^{2} at 1 kc.

Frequency (c./sec.)	Sound level outside (db.)	Sound level inside (db.)	Attenuation (db.)
	Room A		
1000	110	55	55
2000	110	43 ± 2	67
4000	110	42 ± 2	68
6000	110	40 ± 3	70
8000	110	—	>70
10000	110	—	>70
	Room B		
1000	110	53 ± 1	57
2000	110	45 ± 1	65
4000	110	43 ± 2	67

The results, shown in Table 1, are self-explanatory. Since the sounds produced by the main species being investigated contain practically no audio-frequencies below 2000 c./sec. (2 kc.), a screening which attenuates this frequency and above by *not less than* 67 db. (i.e. approximately by a factor of over 3500) was felt to be eminently satisfactory. Thus at an average of 6000 cycles (6 kc.) sound outside the room at 110 db. was reduced inside the room to an intensity less than that produced by the respiration, blood circulation and heart-beats of the observer.

SUMMARY

The design of an inexpensive sound-proof room, virtually sound-proof to audio-frequencies of 2 kc. and above, is described. Since for the purposes of many zoological experiments low frequencies can safely be disregarded, it is felt that on account of cheapness and relative ease of construction, this type of sound-proof room has much to recommend it.

We are greatly indebted to Mr H. R. Humphreys, of the Engineering Division of the British Broadcasting Corporation, and to Mr N. Fleming, of the National Physical Laboratory, for most valuable technical advice on the details of construction. We are also very grateful to the British Broadcasting Corporation for the loan of a Warble Tone Generator and a Dawe Sound Level Meter. Mr J. A. Popple gave indispensable advice and help in measuring the efficiency of the rooms.

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HEARING IN CERTAIN ORTHOPTERA

I. PHYSIOLOGY OF SOUND RECEPTORS

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(*Received 7 June* 1956)

INTRODUCTION

The basic physiology of sound reception in orthopteroid insects, as mediated by tympanal organs and various types of hair receptor, was described by Pumphrey and Rawdon-Smith in a series of papers between 1936 and 1940. In 1940 Autrum published identical conclusions in Germany. Since then, no further physiological data on these organs have been published. Thus at the Paris conference on the acoustics of Orthoptera in 1954, one of the chief drawbacks to discussion was the fact that the physiology of reception was only partially known in half a dozen species, and had never been clearly related to acoustic behaviour in these insects, since neither Pumphrey and Rawdon-Smith nor Autrum had investigated the nervous discharge consequent on stimulation of the sound receptors of their experimental insects by stridulation of the orthopteroid type.

The present papers describe work on the physiology of reception carried out during an investigation of stridulation and associated behaviour in certain Orthoptera.

MATERIALS AND METHODS

A group of four closely related Orthoptera was examined in order to obtain comparative data on sound reception; the insects were adult grasshoppers of the group Acrididae, namely *Stenobothrus lineatus* (Panzer), *Omocestus viridulus* (Linnaeus), *Chorthippus parallelus* (Zetterstedt), and *C. brunneus* (Thunberg) (= *C. bicolor* (Charp)). These insects occur in intermingled 'colonies' on grassland at Imperial College Field Station, Silwood Park, Sunninghill, Berkshire, and their occurrence, general biology and population dynamics have been worked out by Richards & Waloff (1954).

The organs investigated were the tympanal organs, long hair sensillae on the anal cerci and hair sensillae on the thorax and abdomen; the method used was an electro-physiological one similar to that of Pumphrey & Rawdon-Smith (1936*b*). A double-walled copper box, lagged to prevent echo, was used to house the nerve preparation; a short copper tube 5 in. in diameter directed sound stimuli at the preparation through an opening in the side of the box.

* Now at the Anti-Locust Research Centre, London.

The nerve preparation used for each organ is described under the appropriate heading. The recording system was the conventional a.c.-coupled amplifier and double-beam oscilloscope with photographic recording.

Pure tone stimuli were derived either from a B.S.R. oscillator type LO 800A or a specially built beat frequency oscillator (Haskell & Haskell, 1955), the sound being directed at the preparation by loudspeaker as mentioned above. No electrical interference was experienced from the stimulus; the waveform and frequency of the stimulus was monitored by a crystal microphone inside the preparation box feeding an audio-amplifier, whose output could be displayed on an oscilloscope. The intensity of the stimulus was measured by a Dawe sound level meter type 1400C, the microphone of which was mounted inside the preparation box; this enabled the sound pressure at the preparation to be determined in decibels relative to a reference level where 0 db. was equivalent to 0·0002 dyne/cm.^{2}. All measurements of intensity given in this paper are relative to this reference level. The meter gave readings for sounds in the frequency range 50 c./s.–13 kc./s., down to a level 24 db. above the reference level. Sound levels at frequencies between 13 and 20 kc./s. (the limit of the oscillators) were derived by calculation. Although not so accurate as figures from the level meter, these calculated figures agreed well with the curve of frequency/sensitivity as given by the data derived from the meter and so have been allowed to stand.

Pulse stimuli, whose intensity, duration, and rate of repetition could be varied, were obtained by passing the output of a square wave generator through a transformer coupled to the stimulus loudspeaker.

RESULTS

(*a*) *Tympanal organs*

The nerve preparation for tympanal organs described by Pumphrey & Rawdon-Smith (1936*b*) for *Locusta* was first tried but proved to be unsatisfactory owing to the small size of the present insects, and the following method was used. The insect was secured in a block of plasticine ventral side down and the tegmina and wings were cut off. A small sagittal dorsal cut was made in the integument backwards from the pronotum and held open by small wire hooks attached to pins. The section of gut underlying this cut was removed and fat and connective tissue were cleared away to uncover the metathoracic ganglion; the tympanic nerve was then picked up on the electrodes and the preparation and apparatus were checked by giving a short burst of stimulus. The nerve was then severed from the ganglion and the screening box closed. Such preparations maintained their initial sensitivity for long periods, often up to 5 hr. and longer if occasionally moistened with a suitable saline (Pringle, 1938). Decapitation of the insect was not carried out; it caused no change in the nervous discharge and generally led to more rapid desiccation with consequent loss of sensitivity. Care was taken not to puncture or disarrange the air sacs surrounding the tympanal organs more than necessary; but in fact even complete removal had little effect on sensitivity and none on the pattern of nervous discharge.

Determination of the threshold sensitivity at various frequencies was carried out as follows: beginning with low frequencies, the intensity of the stimulus was adjusted until a response was just obtained from the preparation, when the sound level was read on the meter. The frequency of the stimulus was then increased and the process repeated, until the frequency limit of the stimulating oscillators, 20 kc./s., was reached. It is virtually certain, in view of the work of Auger &

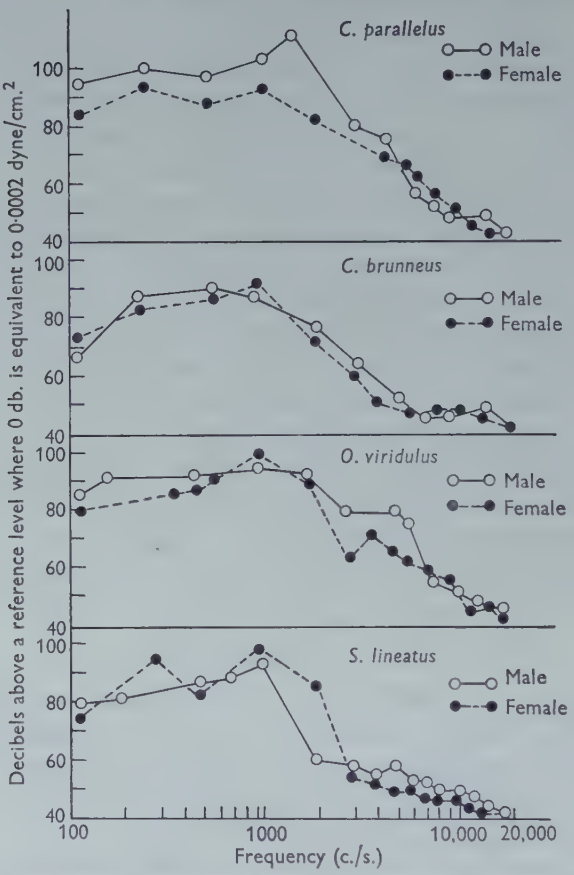

Fig. 1. Graphs showing threshold response of tympanal organs as a function of stimulus frequency in certain Acrididae.

Fessard (1928), that the tympanal organs in Acrididae respond to sound over a much greater frequency range than 0–20 kc./s.; but this range covers all the frequencies found in the natural stridulation of the species, which was all that was necessary for the present study. When one run over the whole frequency range had been completed, the external noise level was measured to ensure that this was too low to interfere with the response of the preparation. This procedure was carried out three times for each individual insect, and the levels for each frequency were then averaged; the readings at all frequencies were found to agree within 5 db. in every case.

Fig. 1 shows graphs relating the threshold intensity in decibels to frequency of a pure tone stimulus for the four species; each graph is the average for three specimens.

The nervous response to a pure tone stimulus between 0 and 20 kc./s. was asynchronous in all cases. Fatigue, equilibration or adaptation were not observed in any case of prolonged stimulation. Under conditions of zero stimulation, a resting discharge was present.

The nervous response to sounds consisting of trains of pulses was also studied.

Fig. 2 shows a series of oscillograms of action potentials in the tympanic nerve of a male *Stenobothrus lineatus* due to stimulation with pulsed sound from the square wave generator; at pulse repetition frequencies between 1 and 90 per second

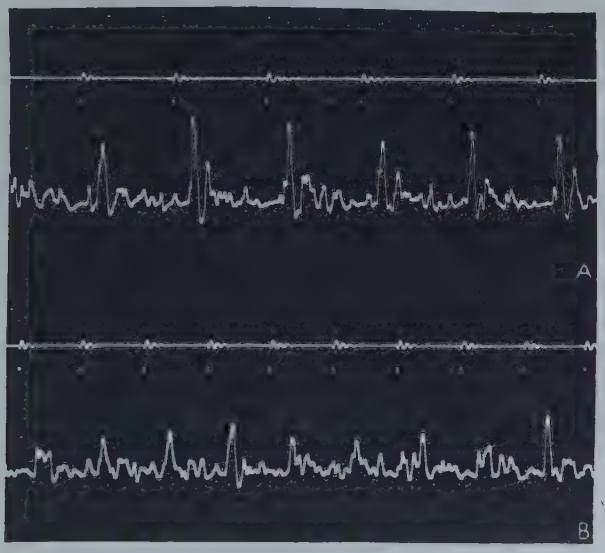

Fig. 2. Response in tympanic nerve of *Stenobothrus lineatus* on stimulation with pulsed sound. Stimulus frequency (A) 15 per sec., (B) 50 per sec.

the nerve discharge is synchronous with the stimulus, although falling in amplitude as the top of the range is approached. Above 90 pulses per second synchronism breaks down, and in all four species this was found to be the case at rates between 90–100 pulses per second.

By measuring the interval between pulses at repetition rates at which asynchronous responses begin to appear, figures for what might be called the 'refractory period for the system' were obtained. These were of the order of 20–30 msec. for all four species, and represent the least interval that must occur between successive sound stimuli to allow them to produce a synchronous discharge in the tympanic nerve.

Although Pumphrey (1940) reports that in *Locusta* the synchronous discharge of volleys at the modulation frequency of the stimulating sound showed the phenomena of 'alternation' and 'equilibration' these were not noted in the present experiments with the frequencies and stimulation times used. Finally it may be stated that

continuous stimulation at subliminal level does not produce discharges by temporal summation.

These experiments were repeated with males and females of all four species, results being identical in all cases. Females were also tested on maturation of the ovaries and just prior to and after oviposition, but the nervous responses at these times were the same as previously described. This is of importance since observations on behaviour associated with stridulation in these species (Haskell, in preparation) shows that at these times important changes in acoustic responses occur.

(*b*) *Long hair sensillae on anal cerci*

The anal cerci of the present species are small, generally of the order of 1 mm. in length, and the number of long hair sensillae on them is also small. Although different in size, the receptors appear identical in external morphology with the long hair sensillae on the anal cerci of the domestic cricket which have been described by Sihler (1924). No histological work on these organs was carried out, but in view of the fact that they exhibit responses similar to those described by Pumphrey & Rawdon-Smith (1936*a*, *b*) for the sensillae of the cricket it is probable they have a similar structure.

The nerve preparation was made by sinking the insect in a block of plasticine, ventral side down, cutting off the tegmina and wings and making a dorsal sagittal cut at the rear end of the abdomen; gut and connective tissue were then removed to reveal the last abdominal ganglion. The nerve to one of the cerci was picked up on the electrodes, the preparation tested, and the nerve cut at the ganglion. In the present species the nerve is very fine and the preparation rapidly loses sensitivity (presumably due to desiccation, although this could not be arrested by the application of a suitable saline).

Fig. 3 A shows the response of these hair sensillae in a male *Omocestus viridulus* to a puff of air directed at them from a small pipette one foot away; this response was common to all four species. The receptors in all species also showed a synchronous discharge to stimulation with pure tones up to 300 c./s. Both these types of response were abolished when the hairs on the cerci were entangled with one another by smearing them with vaseline. No clear evidence of equilibration or frequency halving or doubling was obtained with these preparations.

That the cercal fibres synapse with fibres in the ventral nerve cord was shown by recording from the latter between abdominal ganglia 7 and 8, when spikes of the type associated with giant fibres were produced by stimulation of the sensillae on the cerci. Continuous maximal stimulation of the sensillae produced accommodation in the post-synaptic fibres, and Fig. 3 B shows this effect in a male *O. viridulus*.

On comparing these results with the findings of Pumphrey & Rawdon-Smith as regards the acoustic functions of cercal sensillae in the cricket (1936*b*) and the transmission of impulses from the cercal nerve to the ventral nerve cord in the cockroach (1937), it is considered that the evidence is sufficient to justify the assumption that the long hair sensillae on the anal cerci of the present insects

respond to sounds in the same manner as the sensillae on the anal cerci of crickets and cockroaches and that a similar arrangement of synapses with giant fibres in the ventral cord exists in all these insects. This assumption is supported by the work of Cook (1951) which confirms the existence of a similar system in *Locusta migratoria*, another acridid.

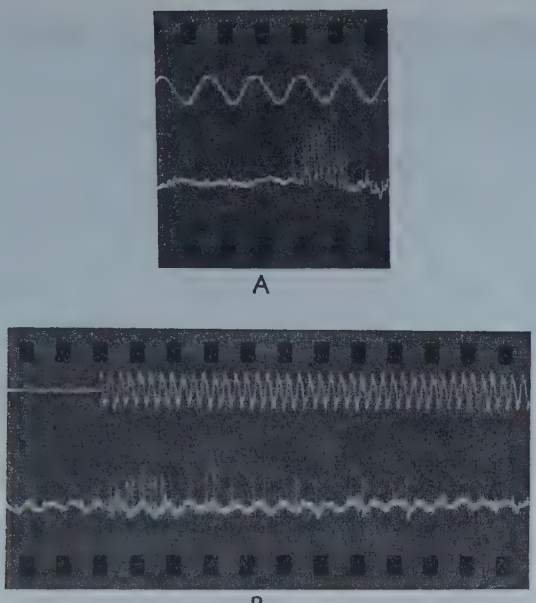

Fig. 3. Responses of hair sensillae of *Omocestus viridulus*. (A) Response to air puff; timing wave 50 c./s. (B) Accommodation of post-synaptic discharge in ventral nerve cord on continuous stimulation of hair sensillae with pure tones.

(*c*) *Hair sensillae on the thorax and abdomen*

Pumphrey & Rawdon-Smith (1936*a*) showed that some unidentified end organs situated in the abdomen of *Locusta migratoria*, with afferent fibres in abdominal segmental nerves, were sensitive to air-borne sounds. In the present species preparations of the second and third tergal nerves of the metathoracic ganglion (2 Tg Nv, 3 Tg Nv, Albrecht's (1953) notation), when severed from the ganglion, gave responses when the insects were exposed to stimulation by air-borne sounds.

Fig. 4 shows electrical responses from the third tergal nerve, left side, of a female *Chorthippus parallelus*, to the recorded song of the male of the same species; trace A shows responses in the normal insect, which were greatly reduced (trace B) when the thorax and second and third abdominal segements were smeared with vaseline. The nature and meaning of the residual activity in the nerves after the sensillae were vaselined will be referred to in the discussion.

It proved impossible to obtain a preparation with one fibre only firing, and thus threshold measurements refer to the sensitivity of a group of sensillae. Because of this, only a few measurements were made at frequencies between 2 and 4 kc./s. when

it was found that over this range, a minimum pressure of 7 dynes/cm.^{2} at the sensillae was necessary to produce a response. The discharge due to the hair receptors was asynchronous with regard to the stimulating sound, but increases in the number of active fibres and in the frequency of discharge followed increases of stimulus intensity. No fatigue was noted after prolonged stimulation.

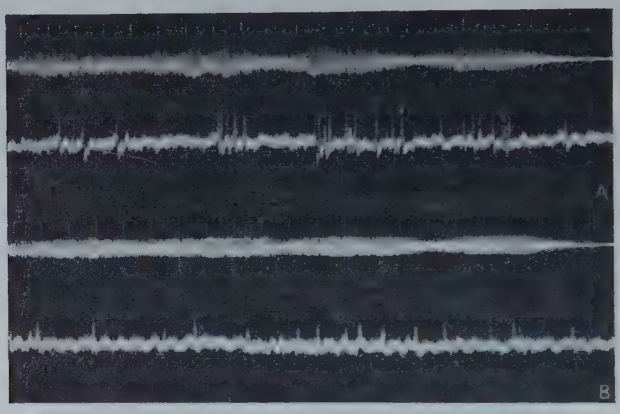

Fig. 4. Responses in tergal nerve of *Chorthippus parallelus*; for explanation see text.

The hair sensillae on the ventral side of the abdomen are in a position to mediate responses to vibration of the substratum, and discharges were elicited in the tergal nerves of all species in response to vibrations of the preparation platform produced by a gentle tap with a rubber-coated glass rod. The vibrations were picked up by a Rothermel V.P. 5 vibration pick-up and displayed on one trace of the oscilloscope. A crystal microphone was placed near the preparation during this experiment, feeding a loudspeaker through an audio-amplifier, to allow the observer to check that air-borne sounds were not being produced by the tapping of the substrate. The response of the hairs to vibration was completely abolished when the abdominal sternites were smeared with vaseline.

DISCUSSION

The results show that in the experimental insects at least three types of receptors exist which are sensitive to sound. Of the three, it seems dubious, however, if the long hair sensillae can be greatly involved in the reception of stridulation, in view of the poor development of the anal cerci, the few hair sensillae found thereon and the restricted frequency range of these receptors. It has been suggested above, that the arrangement of central representation of these sensillae is similar to that of analogous receptors in the cricket, cockroach and migratory locust. In the cockroach stimulation of the cercal sensillae by a puff of air produces the 'evasion response', a burst of rapid locomotor activity, but in the locust this response is poor or absent (Cook, 1951). The anal cerci of *Locusta* are larger and the number of sensillae greater than in any of the species here studied, which suggests that their

effect, if any, in the latter will be small, and they will therefore not be considered further as organs concerned with the reception of stridulation. It is possible that the true function of these organs in seen in the act of mating, during which they are mechanically stimulated by copulatory movements, when they may influence the state of central excitation. Huber (1952) has demonstrated that the cercal sensillae in *Gryllus campestris* must be stimulated for full mating behaviour to be realized in this species.

The remaining receptors sensitive to sound are the tympanal organs and the thoracic and abdominal hair sensillae. The basic physiology of the response of acridid tympanal organs to sound has long been established (Pumphrey & Rawdon-Smith, 1936*a*, *b*, 1939), and in all cases the responses of these organs in the present species conformed to their findings. The sensitivity of these organs is high for such simple structures, and Pumphrey (1950) has pointed out that the resting discharge, present in most invertebrate hearing organs so far investigated, may indicate a regenerative system, whose sensitivity would be greater than any non-regenerative counterpart. This latter system has recently been found to exist in certain lepidopterous tympanal organs (Haskell & Belton, 1956), but no comparative data on sensitivity as between the two types are yet available. The threshold graphs for the four species show there to be little difference in sensitivity between them; this is to be expected considering the similarity in size and structure of the tympanal organs throughout the group.

It must be pointed out that data for threshold sensitivity derived from the graphs of Fig. 1 must be treated with caution. One reason for this is that, if the tympanic system is regenerative, the threshold will change in an irregular manner and any measurement only represents the average sensitivity at the moment of recording. But the most important reason is the fact that of all physical quantities sound intensity is perhaps the most difficult to measure accurately. In experiments such as one described above, done without benefit of a soundproof room, allowances of $\pm 10\%$ at least must be made on all figures. If this figure seems large it must be remembered that the main desideratum in sound measurement is a completely homogeneous medium to avoid refraction, reflexion and absorption, and owing to convection and conduction of heat by air it is very difficult to obtain such conditions in a laboratory. However, the fact that three trials were made in these experiments, in which readings at particular frequencies all agreed within 5 db., gives some guarantee that conditions were reasonably constant over the experimental period.

In the light of the findings of Pumphrey & Rawdon-Smith (1939) on the response of the tympanal organs of *Locusta migratoria* to modulated sound it was to be expected that the nervous discharge consequent on stimulation of the tympanal organs in the present species with pulsed sound would be volleys of spikes synchronous with the pulse repetition frequency of the sound. Fig. 3 clearly shows this; as the limiting pulse frequency is reached the spike height of the resultant discharge is considerably reduced. In the species studied, synchronism broke down at pulse repetition frequencies between 90 and 100 per sec.; this figure is far in excess of any pulse repetition rates found in the natural stridulation of the species.

Turning now to consider the receptors which mediate responses in the segmental nerves we find differing opinions as to the type of sense organ concerned. Pumphrey & Rawdon-Smith (1936*a*) at first considered the responses to originate in the hair sensillae distributed over the body of the locust. Pumphrey, however, in a later paper (1940) inclined to the view that the organs concerned were segmental chordotonal sensillae, the evidence for this modified view being that there only appeared to be two active fibres in the segmental nerves, and the receptors did not fatigue on constant stimulation as do most hair organs. In the present work the pattern of nervous discharge has indicated a variable number of active fibres in the various preparations, ranging from three to five. In some preparations the number of active fibres depended on the intensity of the stimulus, a higher intensity increasing the number of firing fibres as well as increasing the frequency of discharge, which supports the view that the receptors are hair organs. The nearly complete abolition of response in the segmental nerves when test insects were smeared with vaseline also points to hair organs as being the receptors concerned. However, some residual activity was recorded from the nerves after the application of vaseline, and furthermore the receptors did not fatigue with a constant stimulus, all of which evidence supports the view that the responses were due to chordotonal organs. Hughes (1952), recording from the segmental nerves of *Locusta*, found discharges due to acoustic stimuli and also bursts of impulses during respiratory movements of the insect; he concludes that 'some sense organs respond to both the respiratory movements and auditory stimulation, while others are excited by either one or the other'. It thus seems possible that residual activity after application of vaseline in the present insects might be due to the effects of respiratory movements on chordotonal organs, and that hair organs act as the main receptors of acoustic stimuli.

Examination of the abdominal chaetotaxy in the present species supports the view. The 2nd and 3rd tergal nerves of the metathoracic ganglion, from which recordings were made, supply the 2nd and 3rd abdominal segments respectively. On the sterna of these segments there is a fairly large number of hairs approximately 250μ in length; in the normal resting position of the insect these would press against the substratum, and while suited to receive vibrational stimuli would be unlikely to respond to air-borne sounds. The pleura of these segments have short hair sensillae, approximately 50μ in length, distributed over them; these hairs are fairly stiffly articulated. However, at the sternal borders of the pleura a very few of the longer (250μ) hairs are found, with a considerably looser articulation, which move when a current of air is passed over them. These hairs are in a position to respond to air-borne acoustic stimuli, and their relative scarcity may explain the small number of active fibres in the preparations. Although this evidence is suggestive, more detailed work is required to place the matter beyond doubt.

If it be accepted that these hair organs do act as receptors for acoustic stimuli, then their use in nature for mediating responses to natural stridulation in the species under discussion must be greatly restricted. If their maximum sensitivity is of the order of 70–80 db., as suggested by the present findings, then the range

over which they respond to natural stridulation in the field must be measured in centimetres, since data on the sound intensity of stridulation in the present species (Haskell, 1955) indicates that only very close to the emitting insect are such levels reached. It is possible that these organs may be called into play during the courtship behaviour of these acridids, when males and females manoeuvre within a centimetre or so of one another.

Whether or no these hair sensillae respond to air-borne stimuli, the hairs on the sternites certainly mediate the responses to vibration recorded from the segmental nerves, since this response is completely abolished on the application of vaseline to the appropriately situated sensillae.

SUMMARY

1. The physiology of sound reception by tympanal organs, long hair sensillae on the anal cerci, and hair sensillae on the thorax and abdomen has been studied by electrophysiological methods in four species of Acrididae, namely *Stenobothrus lineatus*, *Omocestus viridulis*, *Chorthippus parallelus* and *C. brunneus*.

2. The discharge in the tympanal nerve of all four species on stimulation by pure tones was asynchronous and showed no equilibration or fatigue. Comparative data are given for threshold sensitivity to pure tones for the four species over the range 0–20 kc./s., and shows the sensitivity to be very similar throughout the group.

3. The tympanal organs of all four species responded to pulsed sound with synchronous volleys of spikes up to pulse repetition frequencies between 90 and 100 pulses per second; at higher rates synchronism broke down.

4. Long-hair sensillae on the anal cerci responded to gross air movements with bursts of spikes and to pure tones up to 300 c./s. with a synchronous discharge. The cercal nerves synapse with fibres in the ventral nerve cord, and the post-ganglionic discharges on continuous maximal stimulation of the cercal sensillae show accommodation. It is concluded that these cercal sensillae are similar in response and innervation to those on the cerci of the cricket, cockroach and migratory locust, but that owing to their small number and restricted frequency range they are probably not concerned with reception of natural stridulation.

5. Discharges were evoked in segmental nerves of the third thoracic ganglion in the four species in response to vibration of the substratum and stimulation by air-borne sounds. The sensillae mediating the vibration response are hair sensillae situated on the abdominal sternites.

6. The receptors mediating response to air-borne stimuli may be hair sensillae on the abdominal pleura and sternites, or segmental chordotonal sensillae. The present work indicates that the former are probably the receptors concerned, but further work to elucidate the roles played by the two types of organ is required. Data are given on the response and threshold of the hair organs.

7. The responses of all the sound receptors investigated were independent of age and sex and also, in females, of the state of the ovaries.

I am grateful to Prof. J. W. Munro for encouraging this work at its inception, and to Prof. O. W. Richards for his criticism and advice. I am indebted to the following for the loan of apparatus: Mr Toombs, Department of Electrical Engineering, Imperial College; Dawe Instruments, Ltd., Brentford; Mr Bayard, Director of the Acoustics Section, Admiralty Research Laboratory. I have also profited by advice from and discussion with Dr B. P. Uvarov, Director, Anti-Locust Research Centre, Dr N. Waloff and Mr J. W. Siddorn, Department of Zoology and Applied Entomology, Imperial College of Science and Technology, and Prof. R. J. Pumphrey, F.R.S., Department of Zoology, Liverpool University, who kindly read and criticized the manuscript. Some of the apparatus used in this work was purchased with a grant from the Central Research Fund of the University of London, and the work comprises part of a thesis for the Ph.D. degree of that University.

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HEARING IN CERTAIN ORTHOPTERA

II. THE NATURE OF THE RESPONSE OF CERTAIN RECEPTORS TO NATURAL AND IMITATION STRIDULATION

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INTRODUCTION

Behaviour experiments, to be described elsewhere (Haskell, in preparation), carried out with four species of Acrididae, namely *Stenobothrus lineatus* (Panz.), *Omocestus viridulus* (Linn.), *Chorthippus parallelus* (Zett.) and *C. brunneus* (Thunb.) (= *C. bicolor* Charp), demonstrated their ability in the main to distinguish between each other's songs by purely acoustic criteria. They were, however, unable always to discriminate between their own natural stridulation and imitations of it made either by mouth by the experimenter or electronically. These facts pose a question as to the essential mechanism by which recognition of different songs is accomplished in these insects, and in this connexion it is clearly of importance to investigate the pattern of nervous discharge from the various hearing organs on stimulation by the songs of the four species.

Instrumental analysis of these songs (Haskell, in preparation) reveals that the stridulation may in all cases be considered as being made up of a series of sound pulses. The frequency spectrum and intensity of these constituent pulses varies over fairly wide limits for each species, and also varies, but to a lesser degree, for an individual insect according to external environment and physiological state. The number of pulses strung together to form phrases of the songs may also vary, but the rate of pulse repetition, that is, the pulse frequency, is the least variable quality of all. It therefore seems significant that analysis shows the songs of the species under investigation to be characterized by different pulse repetition frequencies. This is also the case with the songs of cicadas (Pringle, 1954).

The work of Pumphrey & Rawdon-Smith (1939) shows that the discharge in the orthopteroid auditory nerve consists of volleys of spikes at the modulation frequency of the stimulating sound, and this has been confirmed for the present species (Haskell, 1956). It would therefore be reasonable to regard the different pulse repetition rates found in the several songs of the four species as the key to their recognition, since it would presumably be this quality of the stimulating sound which would be signalled by the discharges in the auditory nerves. However,

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various workers have assumed that there is some other intrinsic quality in the songs which aids discrimination. For example, Busnel (1955) and Busnel, Loher & Pasquinelly (1954) have argued that the essential element of effective artificial signals is the presence in them of 'transients'. It follows, since the reception of both artificial and natural signals is mediated by the same mechanism, either that natural signals must also be characterized by transients to be effective, or that the artificial signals used by Busnel included some other feature also present in the natural signal. The present paper describes experiments designed to investigate these possibilities.

MATERIALS AND METHODS

The experimental insects have at least three types of receptors capable of mediating responses to air-borne sounds: the tympanal organs, the long hair sensillae on the anal cerci and the hair receptors on the thorax and abdomen. In a previous paper (Haskell, 1956) the basic physiology of these organs has been described, and this work led to the conclusion that probably only two receptors, the tympanal organs and the hair sensillae on the thorax and abdomen, were likely to be concerned with reception of stridulation. Accordingly, in the present work only these two organs have been investigated. The nerve preparations and recording apparatus were as described in the previous paper (Haskell, 1956).

The principal acoustic stimulus was the natural stridulation of the insects themselves. To avoid electronic intermediary stages, a small polythene cage was devised, with a muslin top, lit and warmed by two small electric bulbs run from a dry battery. Male grasshoppers of the required species, placed in this cage with a supply of grass and allowed time to settle down, stridulated freely; the size of this stimulus source allowed it to be placed where most convenient, even inside the preparation box if necessary. No control of intensity or duration was possible, but in the present experiments this was of no concern. Other stimuli used were tape recordings of the various songs of the four species and also recorded and 'live' imitations of certain songs. These last were made by mouth by the experimenter, and, because of the difficulty of producing a rapid series of sound pulses by mouth, were effectively reduced to two in number, imitations of the normal song of *C. parallelus* and *C. brunneus*. This imitation stridulation was shown by behaviour experiments (Haskell, in preparation) to be capable in appropriate conditions of eliciting the same behaviour pattern from females as the natural song in a high proportion of trials. The intensity of all these forms of stimulus could be checked at the preparation by a sound level meter, and the waveform monitored on an oscilloscope.

RESULTS

(*a*) *Tympanal organs*

These organs, by virtue of their great sensitivity, directional characteristics and ability to respond to modulated signals, must be considered the principal acoustic receptors of the Acrididae. Behaviour experiments on the present species show that normal behaviour in response to acoustic stimuli can be elicited from insects in

which all sound receptors except tympanal organs have been rendered inactive. This behaviour includes discrimination between the songs of several species, thus indicating that the insect can effect discrimination on the evidence supplied by the tympanal organs alone. As pointed out above, there are two main possibilities to be considered; these are that the 'recognition characteristics' of stridulation are to be found in the different pulse frequencies of the various songs, or that some other feature in the songs, such as frequency or waveform, conveys the information on which discrimination is based. In either case the pattern of discharge in the auditory nerve must in some way reflect this information; if the discrimination is based upon changes in the frequency or waveform of the stimulus, it is to be expected that the responses produced in the nerve by two stimuli identical in these respects would themselves be, if not identical, at least very similar. To test this, experimental insects were stimulated by a recording of stridulation arranged to give

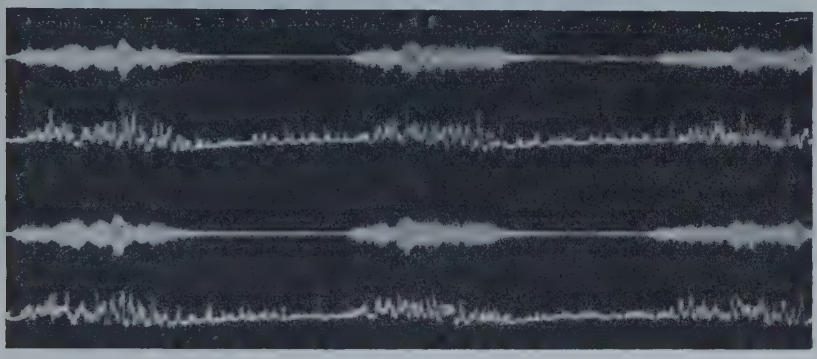

Fig. 1. Response in tympanal nerve of *C. parallelus* to repeated stimulus: for details see text.

repetition of identical signals by using a loop of tape. Action potentials in the tympanal nerve were recorded through several cycles of stimulation, and Fig. 1 shows the response of a female *C. parallelus* to the normal song of a male *C. brunneus*. The upper trace shows the stimulus, which is identical in both records, and the lower trace the corresponding action potentials in the tympanal nerve, which are different in all cases.

In the above results, the stimuli were identical; in the case of natural stimulation the stimulus is variable. The frequency, intensity and waveform may vary from pulse to pulse; this is seen in Fig. 2A which shows the response in the tympanic nerve of a male *Omocestus viridulus* to stimulation by the naturally occurring courtship song of another male. The waveform of each stimulus pulse and the corresponding volley of spikes in the nerve are all different, but the changes in the pattern of nervous discharge bear no simple relationship to the changes in waveform. This variability in the fine structure of nervous discharge was manifested in the responses of both sexes of all four species to stimulation by naturally occurring stridulation. In view of this finding it was to be expected that the response to

artificial imitations of stridulation would also exhibit variability. This is illustrated in Fig. 2B, which shows the response in the tympanic nerve of a male *O. viridulus* to an electronic imitation of the courtship song of the species. The variability of the spike volleys with a constant stimulus should be compared to the variability with natural stimulation in Fig. 2A. The only common factor of the two sets of responses is that the volleys of spikes are synchronous with the pulses of the stimulus which induced them; this finding held throughout the four species for stimulation by all types of song and imitations of song.

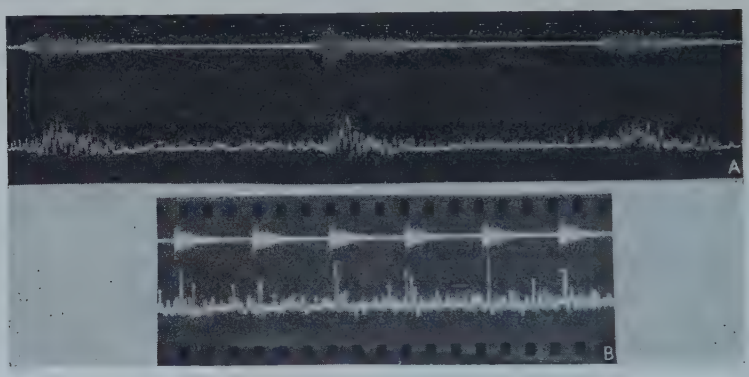

Fig. 2. Responses in tympanic nerve of *Omocestus viridulus*. A, stimulation by natural song; B, stimulation by imitation of song.

(*b*) *Hair sensillae*

The responses of these receptors to both natural and imitation stridulation was investigated. As pointed out in a previous paper (Haskell, 1956) the response pattern obtained in the tergal nerves depends greatly upon the intensity of the stimulus. Fig. 3 shows this effect. The responses are from the third tergal nerve, left side, of a female *Chorthippus parallelus*, the stimulus being the recorded normal song of the male of the same species. The stimulus waveform in both records is the same, but stimulus intensity for record '*b*' is twice that for record '*a*', and the corresponding increase in spike volleys can be seen.

It is interesting to note the apparent synchronism between the song pulses and the volleys of spikes in this record. Experiments showed this to be limited to songs where the pulse rate is very low, that is of the order of 5–6 per second. Response to stimulation by songs with higher pulse rates (e.g. *Omocestus viridulus* normal song) or with a constant 'background noise' (e.g. *Stenobothrus lineatus* normal song) consists of an irregular spike discharge lasting for the duration of the stimulus. The hair sensillae will thus give discharges apparently synchronized with stridulation pulse frequency in two particular cases amongst the present four species examined; these are when stimulated by the normal songs of the males of *Chorthippus brunneus* and *C. parallelus*. This is due to the fact that these songs consist in the case of *brunneus* of a single pulse only, repeated at irregular intervals,

and in the case of *parallelus* of pulses repeated at slow rates up to 5 per second. These special cases are of importance because they have a bearing on the findings of other workers, and they will be referred to again in the discussion.

Generally these hair organs can only signal the duration of a period of supra-liminal stimulation. Experiments with imitation stridulation confirmed these findings in all species.

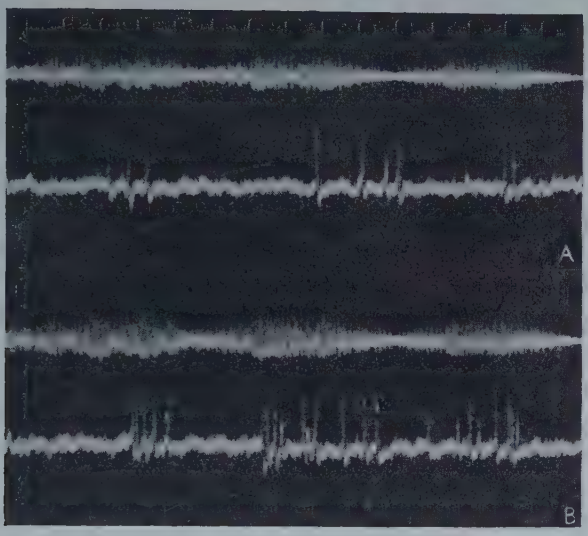

Fig. 3. Discharges in tergal nerves of *Chorthippus parallelus*; for explanation see text.

DISCUSSION

It seems probable from the work of Busnel & Chavasse (1950), M. C. Busnel (1953) and Haskell (in preparation) that all the sounds emitted by the four species here studied lie entirely within the audio-frequency range, and no ultra-sonic component is present, and this discussion is based on this probability. As far as the responses of tympanal organs are concerned, the present work shows that nervous responses to natural stridulation consist of volleys of spikes at the pulse repetition frequency of the stimulus. Since waveform and frequency analyses of natural stridulation have shown that the constituent pulses in a song vary, it was to be expected that the pattern of nervous discharge would alter from pulse to pulse and this was found to be so. This also occurred in nervous responses produced by imitations of stridulation. Thus the only constant feature emerging from these experiments was that in the present species the temporal distribution of spikes in the auditory nerve depended on the pulse frequency of the stimulating sound.

From this, it follows that the key characteristic of the various songs, the characteristic which enables them to be recognized within the species, is the pulse repetition rate. If this hypothesis is true, it should follow that artificial sounds of frequency within the range of the tympanal organs, of liminal intensity and of a pulse modu-

lated type, should be able to initiate the various behaviour patterns known to be related to different songs, provided that the pulse repetition frequency is made to conform to that of these same songs. The behaviour work so far done on the response of Acrididae to artificial signals in general supports this hypothesis. However, Haskell (unpublished results) has noted cases where insects cannot be deceived by imitation stridulation even when all these conditions are satisfied. Also, Busnel and his co-workers in France, after considerable experiment on the responses of certain Acrididae to artificial signals, have come to a different conclusion as to the 'effective element' in sound signals, and it is relevant here to present a short summary of their results to date, taken from the following papers: Busnel & Loher (1953), Busnel *et al.* (1954) and Busnel (1955).

These workers found that a male *C. brunneus* responded to signals from a Galton whistle by stridulating, and could be made to sing alternately with such sounds. If the signal intensity was increased to 75–80 db., the insect responded before the stimulus ceased with a louder song of its own. If the repetition frequency of the whistle signal was increased, the insect sang the 'rivalry' song. In these experiments the intensity of the artificial signal was of the order of 55–70 db., while the natural stridulation intensity is 35–45 db. Males of *C. jucundus* and *C. brunneus* responded in 35–45% of trials to pure tone stimuli between 500 and 20,000 c./s., provided the intensity was 65–70 db. Artificial signals consisting of trains of pulses derived from a square wave generator were also used; each pulse train was of 100 msec. duration. It was found that if the spacing between adjacent pulses was more than 20 msec. the signal was ineffective, but any decrease in the spacing down to 1/16,000 of a second caused the signal to become effective. On the basis of the typical waveforms of the above-mentioned stimuli, which are characterized by sharp rises and falls of intensity at the beginning and end of the signal, Busnel has proposed that the key characteristic in insect sound signals is the existence of 'transients'.

These findings can now be discussed in relation to the results of the present work, and to those of a previous paper (Haskell, 1956). The responses of *C. brunneus* males to Galton whistle and pure tone stimuli is to be expected since, provided the frequency of the signal lies within the frequency range of the tympanal organ and it is of a duration comparable with the natural signal, it will produce discharges in the auditory nerve similar to those caused by stridulation. If the repetition frequency of the artificial signal is increased, so that the signal resembles the 'rivalry' song, so too will the frequency of spike volleys in the auditory nerve be increased, thus providing a physiological basis for the production of the rivalry song in reply.

So far we have only considered the role played by tympanal organs, because, as pointed out in a previous paper (Haskell, 1956), the low sensitivity of the abdominal hair organs precludes their functioning in field conditions except at very short ranges of the order of a few centimetres. However, in the French experiments, a change in response of *C. brunneus* males was observed when the intensity of whistle signals was raised to 75–80 db. At these intensities the hair sensillae will respond to the artificial signal, perhaps producing the modification noted in the response of

the insect. Since it has been shown in behaviour experiments with *C. brunneus* males (Haskell, in preparation), that under certain conditions discharges in tergal nerves produce the same motor responses from insects as similar discharges in the tympanal nerve, it seems possible that the 'supranormal' stridulation responses noted by the French workers to artificial signals of high intensity were a result of the simultaneous stimulation of the tympanal organs and abdominal sensillae, leading to some form of summation in the ganglion centres which altered the normal motor pattern of the behaviour response. It will be remembered that attention was directed above to the special case presented by *C. brunneus* and *C. parallelus* in respect of stimulation of the abdominal sensillae; in these species alone it is probable that discharges in tergal nerves will be synchronous with song pulses. As the French work was largely carried out with *C. brunneus*, involvement of the abdominal sensillae may have induced various 'supranormal' responses, which would not occur in other species. The evidence of the effect of artificial signals on this species must thus be treated cautiously. The findings of Loher (1955, personal communication) that an alteration in intensity of an imitation by mouth of the song of *C. brunneus* produces a change in response from the 'normal' to the 'rivalry' song, may also involve reception by the abdominal hair sensillae in addition to the tympanal organs.

The time relationship found by the French workers to be necessary for the production of effective signals when trains of pulses were used as a stimulus may be explained on the basis of the 'refractory period of the tympanal system' (Haskell, 1956). In *C. brunneus* this was found to be of the order of 20–30 msec. Thus a signal consisting of pulses separated by this amount or more would produce in the auditory nerve a series of action potentials synchronous with each pulse of the stimulus, lasting for the duration of each train of pulses. However, with intervals between pulses of less than the refractory period of 20 msec., synchronism to each pulse breaks down, and the resulting discharge would be a volley of spikes lasting for the duration of each train of pulses, a pattern exactly analogous to the discharge produced by the natural stridulation in *C. brunneus*, and consequently evoking the expected behaviour pattern.

In the light of the above explanations and also the nature of the response of the tympanal organs to stridulation, as shown in the present paper, it seems doubtful whether Busnel's claim that 'les régimes transitoires...constituent...le caractère réactogène essential des signaux acoustiques artificiels' can be made good. Busnel states (1955) that either a sharp increment or decrement of intensity characterizes an effective signal, but it has been found in the present work that responses in tympanal nerves to natural stridulation with such increments or decrements do not show any peculiarities. Furthermore, in behaviour experiments (Haskell, in preparation) responses have been obtained to imitations of stridulation which have varying waveforms not characterized by sharp changes in intensity. It must also be pointed out that most of the French experiments on artificial signals have concerned themselves with the reactions of *C. brunneus*, in which the normal song consists of single irregular pulses of sound. Thus almost any short sound, provided it is in the

right frequency range and of sufficient duration, will produce a discharge in the auditory nerve in this insect similar to that caused by the natural signal, and it is therefore not surprising that Busnel observed a reaction from *C. brunneus* to the starting and stopping of a camera motor (Busnel *et al.* 1954) because the oscillogram of that sound shows it to have all the necessary characteristics for producing a normal discharge in the auditory nerve of the insect.

The preceding argument against the significance of transients is weakened by the fact that in the present experiments records were made from the whole nerve and not from single fibres. It is possible that discharges in single fibres reflect the presence of transients in the signal and afford the insects further powers of discrimination. But if this is so, it would surely be expected that some indication of this would have been noticed during the investigation of the fifty-odd preparations examined in the course of the present work. Pumphrey (1940) also dismisses this possibility as 'unlikely' and describes some experiments by himself and Rawdon-Smith bearing on the problem. Nevertheless, until single fibre preparations have been examined, the possibility cannot be entirely dismissed. Moreover, it has been pointed out by Pumphrey (personal communication) that the responses to the natural signals differ *as a class* from the responses to artificial signals which begin abruptly (see Fig. 2). The significance of this in the subsequent behaviour of the insect can only be investigated when more is known of the method of integration of the signals.

The data emerging from the French experiments support the hypothesis previously mentioned, that the rhythm of repetition of the pulses in a song is the effective character for recognition. As far as sensory apparatus for reception of sound is concerned all the insects studied are identically equipped, and the auditory nerves of all species will carry the same temporal pattern of discharge when exposed to the same sound stimuli; discrimination must therefore be a central process, and probably an innate one. Unfortunately no data are available at present on the central representation or synapses of the afferent fibres from the tympanal organs or abdominal sensillae, although it is clear that some neural organization of a high order is required to integrate the acoustic signals and mediate the complex behaviour associated with stridulation.

It may be useful to suggest that the problem could be examined in relation to the 'Reafferenzprincip' of von Holst & Mittelstaedt (1950). The motor movements necessary to produce stridulation will leave an 'Efferenzkopie' in the central nervous system, and the 'Afferenz' resulting from stimulation by song will be compared with this, thus enabling discrimination between the songs to be realized. On this hypothesis, the simpler the song, the simpler the 'Efferenzkopie', with a consequent reduction in the ability of the central nervous system to distinguish between natural and imitation stridulation; on this basis we can understand why *C. brunneus* with its very simple one-pulse song will respond to a wide variety of imitation signals, which is not the case in insects with more complicated song patterns, such as *Stenobothrus lineatus*. However, both in the French experiments referred to earlier, and in more recent behaviour work (Haskell, in preparation) a

certain proportion of *Chorthippus brunneus* males and females clearly distinguished between natural and imitation stridulation.

What was the basis for discrimination? Loher (1955, personal communication) takes the view that besides the rhythm of the signal, and the existence of transients therein, intensity variation plays a part in discrimination. While this may be so during courtship or rivalry behaviour, when insects are within a few centimetres of each other, it is difficult to imagine it affecting responses to 'normal' song in the field, where, as has been shown (Haskell, 1955) intensity fluctuations of great magnitude are experienced due to micro-climatological conditions. Pumphrey (1955) is of the opinion that the power of discrimination depends in some way on the functional importance of the stimulus situation. This means in effect that the insects use other features of the situation, besides acoustic ones, to aid in song discrimination, and that other sensory modes play a part in initiating the response. Jacobs (1953) states that specific recognition in *Stenobothrus* is partly visual, and in view of the work of Norris (1954) it is apparent that olfactory stimuli may play some part in mating in certain Acrididae. Learning may also be concerned in this behaviour, and in this connexion the work of Regen (1926) with *Pholidoptera* (= *Thamnotrizon*) must be remembered. However, while not entirely excluding the possibility that song characteristics other than pulse repetition frequency may play their part, the present work shows that a satisfactory physiological mechanism exists in the four species for song discrimination based on this pulse repetition frequency, and this may perhaps serve as a working hypothesis for the further behaviour work which is obviously required.

SUMMARY

1. The pattern of nervous discharge from tympanal organs and abdominal hair sensillae resulting from stimulation by natural and imitation stridulation has been studied in four species of Acrididae, namely *Stenobothrus lineatus*, *Omocestus viridulus*, *Chorthippus parallelus* and *C. brunneus*.

2. Experiments with tympanal organs stimulated repeatedly with identical sound pulses show that the resultant volleys of spikes, although synchronous with the pulse frequency of the stimulus, were variable in themselves, and that this variability was random, bearing no simple relationship to any quality of the stimulus. This variability in the structure of the impulse bursts was manifested with both natural and imitation stridulation.

3. The response of abdominal hair sensillae showed them to be capable of signalling the duration of supra-liminal stimuli, and responses signalling pulse repetition frequencies were limited to cases where the pulse rates did not exceed 5 per second. In the present species, this meant that only the normal songs of *C. brunneus* and *C. parallelus* would produce impulse bursts in tergal nerves synchronous with the pulse frequency of the stimulus.

4. On the basis of this evidence it is adduced that the characteristic of stridulation which enables inter-specific recognition to be effected is the pulse repetition rate of the songs.

5. Comparative data from the four species show that the auditory receptors throughout are very similar, and thus the pattern of nervous discharge will be, in its essential element of the rate of impulse burst, the same for all species when exposed to the same stimulus. The discrimination process must thus be a central one, and it is suggested that this problem might be examined in relation to the 'Reafferenzprincip' of von Holst & Mittelstaedt (1950).

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A STUDY OF OVARIAL DEVELOPMENT AND ITS RELATION TO ADULT NUTRITION IN THE BLOWFLY *PROTOPHORMIA TERRAE-NOVAE* (R.D.)

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I. INTRODUCTION

In recent years considerable attention has been directed to the hormonal control of insect growth and, in particular, to those hormones which affect ovarial development. In contrast, although the nutritional requirements for growth of the immature stages of several insects have been investigated, comparatively little is known of the food requirements for ovarial growth specifically.

In the blowfly maturation of the ovaries is controlled by an endocrine complex including corpus allatum, corpus cardiacum and median neurosecretory cells of the brain (Thomsen, 1952). Evidence is also accumulating to show that the corpus allatum produces hormones which influence both protein and carbohydrate metabolism (Thomsen, 1942, 1949, 1952; Pfeiffer, 1945).

On the maintenance diet of sucrose and water the adult female blowfly never lays eggs; ovarial growth ceases at an early stage, although normal changes from pupal to adult fat body and from ring gland to corpus allatum occur. When meat is added to the diet of such flies growth of the ovaries, fat body and corpus allatum is resumed immediately and the ovaries mature a few days later. Since both corpus allatum and the fat body undergo further growth on the complete diet, it is probable on these grounds alone that their functions concern reproduction. Fraenkel (1940) showed that the sucrose of the maintenance diet could be replaced by certain other sugars or polyhydric alcohols but not by protein. The additional requirements for ovarial maturation have been only partly investigated. Hobson (1938) showed that at least two meat meals (approx. 30 ml. beef liver juice) were required for each oviposition and that blood serum + Marmite, but not serum alone, could replace meat. Dorman, Hale & Hoskins (1938) found that meat could not be replaced by casein + butter + yeast, by soluble casein, by blood albumen, or by Lemco beef extract, and concluded that both protein and unidentified accessory substances were required. Banki (1937) was able to rear both larvae and adults on the following mixture: agar, sugar, peptone extract, dried yeast extract, olive oil, cholesterol, ascorbic acid and vitamins A and D. He did not differentiate between active and non-active constituents but found that vitamin E was not required for growth of either larva or adult. Rasso & Fraenkel (1954) have undertaken the most recent investigation. Using *Phormia regina* (Meig.) they report that eggs may be produced with diets containing foods such as casein, casein enzymic hydrolysate, egg

albumen, yeast extract, etc. Proteins must be in soluble form; dialysis reduced the efficacy of sodium caseinate. Better ovarial growth with these diets was obtained by the addition of a mixture of B vitamins and minerals. Choline, nicotinic acid and potassium phosphate were the most effective of the added substances. Synthetic diets never gave such good development as liver. The amino-acid requirements for growth have been investigated in several other insects, e.g. *Tribolium confusum* (Lemonde & Bernhard, 1951), *Blatta germanica* (House, 1949), *Drosophila melanogaster* (Schultz, St Lawrence & Newmeyer, 1946) and *Apis mellifica* (De Groot, 1953); nothing, however, is known of the amino-acid requirement of the blowfly.

In the work here reported the normal development of the ovaries of the blowfly has been studied, and a general investigation made of the food requirements for ovarial growth in adults maintained on a diet of sucrose and water. Although sterile conditions were not used, the risk of bacterial contamination of food was decreased by a strict feeding procedure. It is believed that such bacterial contamination and the use of insufficiently controlled and, in some cases solid, foods may explain the conflicting accounts of former investigators.

II. MATERIALS AND METHODS

(*a*) *Care of stock. Protophormia terrae-novae* were used throughout. Adults were reared under conditions of continuous light, relative humidity 70–80%, and temperature 25° C. Each day, newly laid eggs were transferred to fresh meat in a small plastic dish covered with muslin. The resultant larvae were fed daily with small amounts of whale-meat and were kept moist with wet cotton-wool wrapped in fine muslin. On the sixth day the larvae and meat were transferred to Petri dishes on sand: the migrating prepupae then moved to the clean dry sand below in which they pupated. In-breeding was not controlled and the flies emerging on one day came from only two to four batches of eggs. 200–300 closely related flies were obtained daily and kept in 7 lb. glass jars until required.

(*b*) *Experimental procedure.* Unless otherwise stated, newly emerged female flies were used for experiments. They were anaesthetized lightly with ether and then sexed. Random groups of ten females were marked individually with paint and weighed. Each group of ten was kept in a 7 lb. glass jar covered with Tigron (plastic muslin) and carpeted with filter-paper. All flies were supplied with solid sucrose in crucibles.

In each experiment at least three units of ten flies were used. These flies were treated similarly except in the type of food given which was as follows:

(1) The sucrose control where flies were given a constant supply of water in 5 ml. flasks with cotton-wool wicks. They were given no food other than the sucrose.

(2) The whale-meat control where flies were given a liquid preparation[1] of whale-meat at specific times, and after the last feed were given water as in (1).

[1] Measured quantities of whale-meat and water homogenized in a Waring Blendor and strained through butter muslin. This is referred to in the text as 'whale-meat mixture'.

(3) The experimental jar where flies were given a different type of liquid food which, except where stated, was of a similar protein concentration to the whale-meat control.

A large number of jars with different foods was generally included in this last section, each of which could be compared with another. The two control jars checked the size and activity of the flies and the suitability of the experimental conditions. Where it was necessary to use more than ten flies the jars were duplicated.

For test feeding only liquid was given. It was spread from 1 ml. pipettes over the surface of the muslin cover and protected from evaporation with an inverted Petri dish. Light above the jars attracted the flies to this surface and they fed easily from the film of liquid and the hanging droplets. Spilt food dried quickly on the filter-paper floor, and after 2 hr. the stale food was removed by exchanging the muslin cover. Stock food was refrigerated. In this way the effects of prolonged bacterial contamination of the food were avoided. Flies were given at least eight 2 hr. feeding periods (meals) during the first 7 days. After the last meal flies were again anaesthetized and weighed On return to the jar they were given sucrose and water continuously until dissection. The increased weight of the flies during the feeding period showed that an adequate amount of food had been ingested by the majority in this time. No adverse after effect of ether was discovered.

All flies of one set were killed for dissection on the same day (this was always between 8 and 14 days after emergence) and dissected under Ringer's solution within an hour. The condition of the crop contents, ovaries and fat body of each fly was recorded and the size of each ovary was measured. A micrometer eyepiece was used to measure the dimensions of the ovaries which were spread out flat on a slide. Two diameters at right angles, and the length of the largest egg chamber, were measured. Owing to the regular compact shape of the ovary, the product of these three figures gave a value which, although having no absolute meaning, was roughly proportional to the volume of the ovary. This value was used for finding the mean ovarial size of each fly and each group of flies, thus providing a basis for statistical work. Fat body was classed as pupal or adult, and the latter graded by eye into grades 1–4 according to size, 1 being the smallest.

(*c*) *The effects of experimental conditions.* The following were found to be critical and were kept constant throughout each experiment: number of flies per jar; size of jar; light intensity; number of meals and the interval between them; concentration of amino-nitrogen in the food (protein or amino-acid); solubility of food; the time between last feeding and dissection.

Certain other factors were found to have no effect on ovarial development. These were: the relative humidity, if above 55% R.H., the initial age of the flies at commencement of feeding tests if previously maintained on sucrose and water, and if less than 2 weeks; the larval diet; the diet of the preceding six generations of larvae and adults; the weight of the fly at emergence; the presence of males which encouraged laying, but not growth, of the eggs.

III. RESULTS

(*a*) *Stages in ovarial development*

The ovaries in the blowfly are of the polytrophic type containing roughly 110–180 ovarioles. Only one egg from each ovariole matures at one time, and all the eggs in one fly are at the same stage of development. Five stages of ovarial development have been established and defined as follows (see also Figs. 1 and 2):

Stage 1. At emergence the ovary is small, white and opaque, the ovarioles adhere closely, and the primary egg chamber is not clearly differentiated from the germarium.

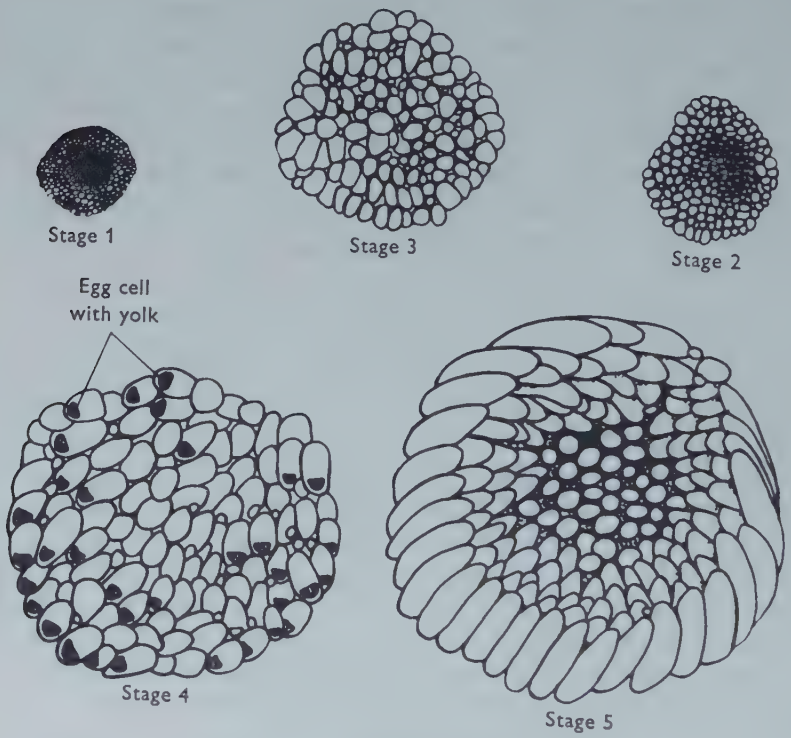

Fig. 1. Whole ovaries to show stages in ovarial development. (× 15.)

Stage 2. The ovary is more translucent. Primary egg chambers are clearly differentiated from the germarium and nurse cells are visible at high magnification.

Stage 3. The primary egg chambers are enlarged, spherical and adhering less closely, giving a bubbly appearance to the ovary.

Stage 4. The primary egg chambers are elliptical, due to growth of both the nurse cells and the egg. The latter contains yolk and is often crescent shaped and generally larger than the nurse cells. Secondary egg chambers have reached stage 2.

Stage 5. The mature ovary is very large. Nurse cells have disappeared and the long curved cylindrical egg fills the primary egg chamber and is fully yolked. The yolk may be quite opaque (*O*) or less dense and partly translucent (*P*).

The differences between stages 1, 2 and 3 are not clear-cut, but the size of the ovaries at each stage is characteristic as shown in table 1.

Table 1

Stage of ovary	Range of length of egg*	Range of volume of ovary*
1	10– 22	60– 300
2	20– 35	200– 2,200
3	30– 45	2,000– 4,000
4	40–120	2,500–11,000
5	120–200	10,000–60,000

* Arbitrary units. 1 mm. ≡ 145 units.

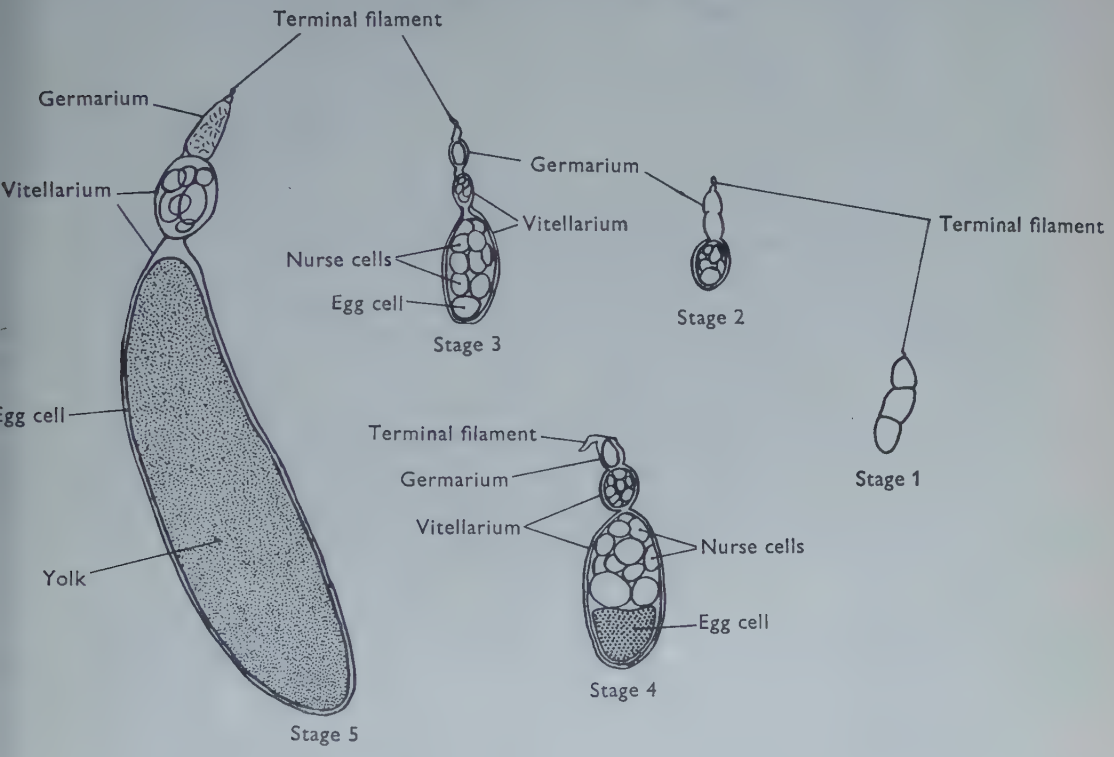

Fig. 2. Single ovarioles at each ovarial stage. (*c.* × 50.)

(*b*) *Growth in the control flies*

Normal development of the adult fly was observed in a large batch of flies kept under uniform conditions as described.

(i) *Sucrose controls* (Fig. 3). In sucrose-fed flies growth of the adult fat body, development of the ovaries to stage 2 and the disappearance of the pupal fat body occur simultaneously during the first 4 days following emergence. Little further change occurs for about 2 weeks, by which time both depletion of fat body tissue and decrease in ovarial size become noticeable.

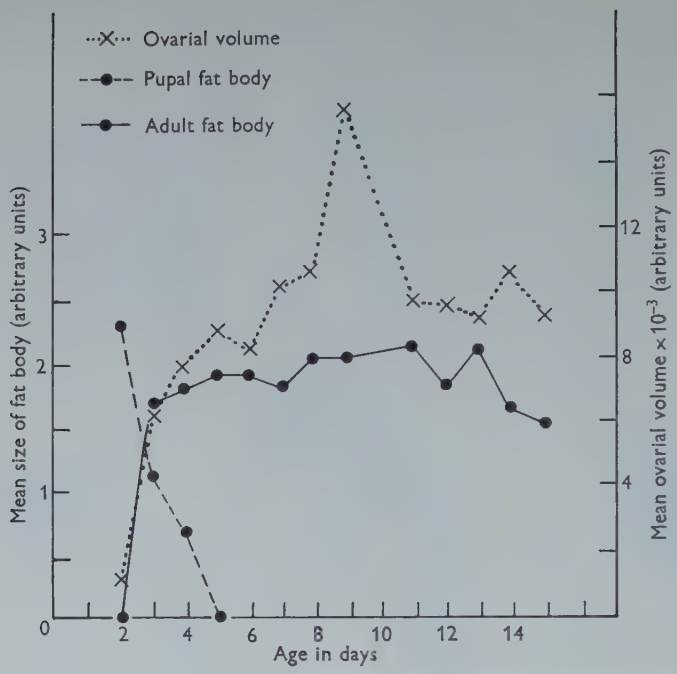

Fig. 3. Sucrose controls (diet of sucrose and water). Graph to show relation between age of fly and growth of ovaries and fat body.

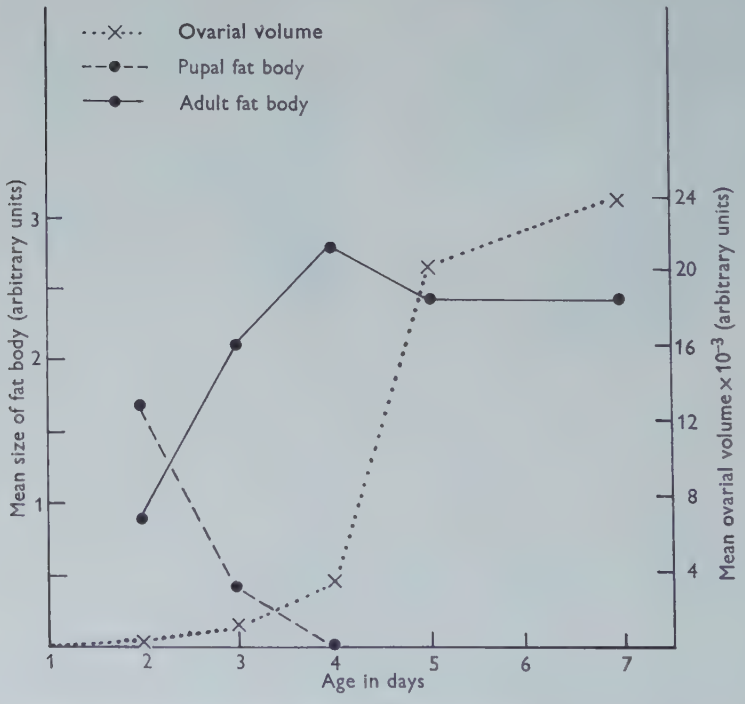

Fig. 4. Whale-meat controls (diet of whale-meat, sucrose and water). Graph to show relation between age of fly and growth of ovaries and fat body.

(ii) *Whale-meat controls* (Fig. 4). In whale-meat fed flies, similar changes in fat body and ovaries have taken place by the third day of adult life. Pupal fat body persists to the third day only, undoubtedly because the basal metabolic rate has been much increased by ingestion of meat. Both ovaries and fat body then undergo very rapid growth and the complete ovarial maturation is accomplished within 5 days. The striking increase in ovarial size accompanying yolk deposition takes place in one day. The speed of yolk formation indicates that there is a large readily mobilized food reserve built up. This is probably greater than can be provided from either the haemolymph or direct absorption of food from the gut. Since the fat body is always large at the onset of yolk formation and has diminished significantly when yolk formation is complete, it is evident that a transfer of material from the fat body to the ovary occurs during this time.

(*c*) *The effects of food on ovarial development*

(i) *The type and amount of food taken*

Normally adult flies can only ingest solid food in the form of very fine particles, although a few foods, e.g. sucrose, are dissolved by the salivary fluid ejected during feeding. All foods employed in this work were used in the form of clear solution, fine suspension or homogenized mixture such as is produced by the Waring Blendor. Dissection disclosed the presence of food in the crop immediately after feeding and up to 7 days later. The presence of coloured food in the crop at autopsy indicated adequate food intake, whilst the presence of congealed meat juice or milk a week after ingestion showed that extensive digestion of protein did not occur in the crop which appears to act chiefly as a store for both food and water. Since ingestion of much water may retard digestion by diluting the food, flies were not supplied with water until experimental feeding was complete.

Each fly was weighed before and after the complete feeding period. The difference in weight was used to express its food intake and results showed (Table 2) that this amount increased with the number of meals. Taste was also important. Table 3 shows that Marmite-containing foods were preferred to others, although sufficient amounts of even less acceptable foods were taken. Minor inaccuracies in this figure are caused by regurgitation, defaecation, water loss, metabolic activity, and also variation due to unequal growth of the ovaries and fat body. The variation in food intake and ovarial growth of individual flies, all of which had ingested considerable amounts of whale-meat juice, is shown in Table 4. In these cases ovarial maturation could not be related to food intake since all the flies had taken in sufficient food; where no ovarial maturation occurred the cause of non-development must be inherent in the fly.

The usual range of weight increase per fly during feeding in any one batch of flies was 16–40 mg. By feeding successive dilutions of a whale-meat juice it was shown that a solution containing 1% protein was the lowest to give satisfactory ovarial growth under standard conditions (Fig. 5). One fly therefore requires not more than 0·4 mg. protein for ovarial maturation. Since most experimental

foods contained at least 5% protein or amino-acid equivalent, sufficient quantity was supplied in 8 ml. food. The mean weight increase during feeding in any one set of flies was nearly always 16 mg., thus giving a safety margin which was considered adequate.

Table 2. *Relation between food intake and number of meals*

Food given	Mean increase in weight after feeding (mg.)				
	1 meal	2 meals	4 meals	7 meals	10 meals
Whale-meat mixture	8·0 (3·5)*	9·2 (6·9)	22·7 (4·0)	34·0 (7·7)	43·0 (10·5)
Horse serum & Marmite	5·4 (3·7)	13·2 (4·5)	24·2 (7·2)	19·0 (9·5)	23·5 (10·4)

* Bracketed figures indicate standard deviation (±).

Table 3. *Experiment to show that gelatin, additional amino-acids and Marmite are all required for yolk formation. The preference of the flies for foods containing Marmite is also shown*

Food given	Mean increase in weight	Mean ovarial volume	No. of flies
Sucrose control	16	349	7
G	18	564	11
M	27	1,017	10
A	31	1,542	8
G+M	32	2,955	12
G+A	14	974	10
M+A	28	612	8
A+G+M	33*	17,944	12
Whale-meat control	46*	26,642	10

* Flies had mature ovaries.
G = 10% gelatin hydrolysate (4 hr. autoclave 120° C.); A = amino-acid mixture A; M = Marmite solution 4%.

Table 4. *One typical set of results for flies fed on whale-meat juice and sucrose to show the variation in the response of individuals*

Weight on emergence (mg.)	Weight ingested during feeding period (mg.)	Fat body size	Mean ovarial volume	Ovarial stage
38	16	2	2,220	5·0
36	19	2	34,143	5·0
35	21	1	3,864	2
34	23	2	1,994	3
29	24	2	29,817	5·0
34	25	1	229	1
36	26	2	3,864	4
34	26	2	5,876	5·0
38	27	1	26,558	5·0
33	35	2	29,974	5·0
37	36	1	33,554	5·0

(ii) *Natural foods*

During preliminary work many natural foods were tested, as shown in Table 5. Of these, whale-meat remained the most effective, although flies with mature ovaries were also found when they were fed on crude serum albumen, crude egg albumen and concentrated Marmite solution. Other protein-containing foods such as milk, horse serum, and prawn muscle, gave only slight enlargement of the ovaries without yolk formation, while gelatin hydrolysate gave no ovarial growth. When any of the above-mentioned foods, all of high protein content, were combined with small amounts of Marmite, good ovarial development resulted, except with gelatin hydrolysate. Thus both 'accessory substances' present in Marmite and protein are required for yolk formation.

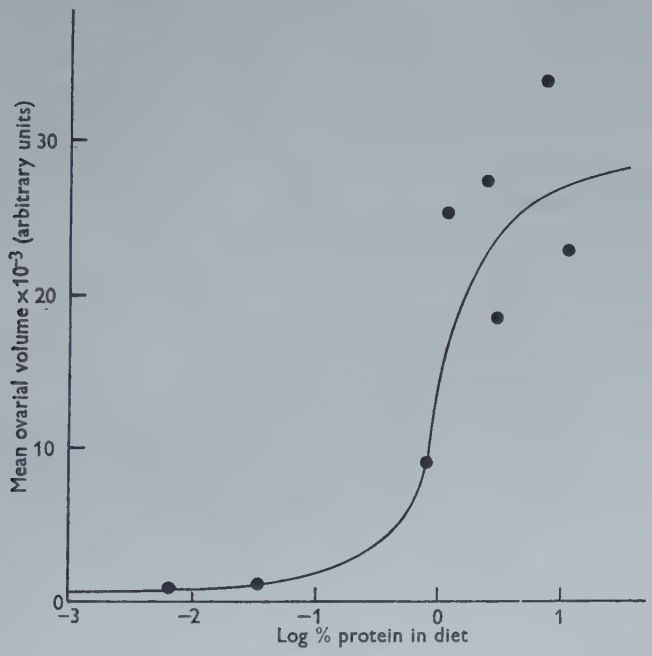

Fig. 5. Graph to show relation between the concentration of whale-meat juice in the food and resultant growth of the ovaries.

(iii) *Preparation of whale-meat*

(*a*) *Boiled whale-meat juice.* Whale-meat juice (the liquid drained from the meat) which had been boiled for 2 hr. was still an effective diet, although the ovaries produced were smaller than with unboiled meat juice (Table 6). This reduction in size was attributed to a reduced intake of protein because of coagulation and not to destruction of accessory factors. The boiled filtrate of whale-meat mixture produced only slight ovarial enlargement when given alone, and, as was shown by combining it with milk and Marmite, was not a good source either of protein or of the accessory factors.

Table 5. *To show the effect of adding small amounts of Marmite to some natural foods containing protein*

Food given	No. of meals given	Age at autopsy in days	Mean size of ovaries
Prawn muscle	Solid meat for 2 days	7	994
Whale-meat control		7	34,225
Sucrose control	0	7	695
Gelatin hydrolysate	10	14	392
Gelatin hydrolysate + Marmite 4 %	10	14	1,929
Whale-meat mixture control	10	14	26,642*
Sucrose control	10	14	285
Horse serum	4	8	2,110
Horse serum + Marmite 4 %	4	8	17,982*
Whale-meat control	4	8	41,179*
Sucrose control	4	8	1,336
Milk	4	8	1,064
Milk + Marmite 4 %	4	8	24,650*
Whale-meat control	4	8	29,060*
Sucrose control	4	8	307
Egg albumen	5	14	2,769
Egg albumen + Marmite 4 %	5	14	15,096*
Serum albumen	5	14	3,202
Serum albumen + Marmite 4 %	5	14	21,562*
Whale-meat control	5	14	17,934*
Sucrose control	5	14	882

* Some flies had mature ovaries.

Egg albumen and serum albumen were reconstituted from dried preparations to 7 % solutions. Gelatin hydrolysate = 10 % gelatin autoclaved 4 hr., 120° C.

Table 6. *Whale-meat preparations*

Food given	Mean size		No. of flies	
	Fat body	Ovary	Total	Ovary, stage 5
Whale-meat juice	3·3	45,868	8	8
Boiled whale-meat juice	3·2	22,405	9	8
Sucrose control	2·1	381	8	—
Sucrose control	2·0	849	9	—
Whale-meat mixture	1·9	26,295	10	9
Marmite 4 %	2·8	1,528	8	—
Boiled meat filtrate + Marmite	2·2	5,808	10	1
Boiled meat filtrate + milk	2·2	22,706	9	7
Boiled meat filtrate	2·5	1,364	4	—
Dialysable fraction	2·0	1,315	6	—
Non-dialysable fraction	3·1	2,092	6	—
Dialysable + non-dialysable fractions	2·5	15,055	10	3
*Dialysable fraction + gelatin + amino-acids	3·0	10,379	2	1
Gelatin + amino-acids	3·1	1,288	9	—
Whale-meat control	2·0	16,679	3	2
Sucrose control	2·0	1,193	5	—

* Inadequate numbers, but because the result was positive it is of significance.

Gelatin was given as the hydrolysate (4 hr. autoclaving, 120° C.) of a 10 % solution. Amino-acids = amino-acid mixture A, see Table 7, footnote. The meat 'filtrate' was made by boiling whale-meat mixture for ½ hr. and then filtering through Whatman's paper no. 1.

(*b*) *Dialysis of whale-meat mixture.* Dialysis of whale-meat mixture (Table 6) resulted in the separation of two active fractions. Moreover, recombination of the fractions in the original proportions gave satisfactory ovarial growth. Gelatin and amino-acids could be substituted only for the non-dialysable fraction. The 'accessory factors' were therefore dialysable.

(iv) *Protein and amino-acid requirements*

(*a*) *The need for tryptophane.* Using Marmite in 4% solution—this containing only small amounts of amino-acids—as a source of accessory factors, the efficacy of many protein and amino-acid mixtures was tested, although it was not possible to eliminate any one amino-acid completely from the diet. Table 7 shows results obtained by giving Marmite + gelatin steam hydrolysate and various combinations of seven amino-acids. Absence of tryptophane prevented yolk formation; absence of any of the remaining six amino-acids was without effect (sufficient may have been provided in the gelatin and Marmite). When tryptophane alone was added to the Marmite and gelatin mixture, ovarial growth was as good as when the seven amino-acids were added. Tryptophane is therefore essential to yolk formation.

Table 7. *To show that a gelatin and Marmite* (4%) *solution is deficient only in tryptophane*

Food given	Mean size		No. of flies	
	Fat body	Ovary	Total	Ovary, stage 5
G+M+A	2·2	24,769	4	4
G+M+A−cystine and methionine	3·3	17,742	6	4
G+M+A−hydroxyproline and serine	2·3	16,899	9	5
G+M+A−tryptophane	2·1	1,519	5	—
G+M+A−tyrosine and valine	Flies accidentally killed			
G+A	2·3	1,704	7	—
Whale-meat control	2·1	18,368	8	3
Sucrose control	2·0	847	8	—
G+M+A	2·1	21,890	42	24
G+M+tryptophane	2·0	17,127	39	17
Whale-meat control	2·0	34,848	21	20
Sucrose control	1·3	978	10	—

G = gelatin hydrolysate 10%; M = Marmite 4%; A = amino-acid mixture containing cystine, methionine, hydroxyproline, serine, tryptophane, tyrosine and valine.

(*b*) *The dialysis of partial hydrolysates of gelatin and the importance of the proportions of amino-acids present.* A partial hydrolysate of gelatin obtained by cooking a 10% solution of gelatin in a steam autoclave for 4 hr. at 120° C. was dialysed through a cellophane membrane. The two fractions obtained were reduced to the equivalent of a 10% solution by boiling. These two fractions were given to the flies in various proportions together with 4% Marmite and L-tryptophane as shown in Table 8. The non-dialysable fraction gave good ovarial development, while the dialysable fraction gave only slight enlargement of the ovaries with no

yolk formation; bu when the two mixtures were combined in equal proportions ovarial growth was no better than with the dialysable fraction alone. When the two fractions were combined in the proportions in which they occur in gelatin ovarial growth was again good, although the total amount of amino-acids was less than that of the ineffective mixture. The relative amounts of amino-acids in the diet are therefore critical.

Table 8. *The effect of dialysis on a partial hydrolysate of gelatin and the importance of the proportions of amino-acids present in the diet*

Food given	Mean size		No. of flies	
	Fat body	Ovary	Total	Ovary, stage 5
Non-dialysable fraction 10 %	2·1	20,016	35	17
Dialysable fraction 10 %	2·1	3,462	26	1
Non-dialysable 10 % + dialysable 10 % fractions	2·3	4,140	30	5
Non-dialysable 6·4 % + dialysable 3·6 % fractions	2·2	13,735	37	13
Whale-meat mixture 5 % control	2·2	16,049	9	4
Sucrose control	1·8	835	10	—

The percentages given are the proportions, in terms of protein equivalent, appearing in the final diet.

The fractions named are the dialysable and non-dialysable fractions respectively of a 10 % gelatin solution which had been autoclaved for 4 hr. at 120° C.

Marmite (4 %) and *l*-tryptophane were added to all diets containing gelatin preparations.

Preliminary chromatographic analysis of the amino-acid content of each fraction was made by a paper chromatographic method (Consden, Gordon & Martin, 1944). The fractions were prepared for chromatography by acid hydrolysis, absorption by Zeocarb (285), elution with ammonia and concentration by boiling. The chromatograms showed that while phenylalanine was found only in the dialysable fraction, the following amino-acids were found in both fractions: hydroxy-proline, proline, alanine, glycine, aspartic acid, glutamic acid, valine, leucine, isoleucine; eight other amino-acids may have been present but were detected in neither fraction. The two fractions are therefore different in composition, but whether the inefficiency of the dialysable fraction was due to the lack of vital amino-acids or merely to unsuitable proportions of those present could not be here concluded. Opportunity of resolving this question was not available.

(*c*) *Replacement of protein by pure amino-acid mixtures.* Replacement of protein in the diet by equivalent mixtures of amino-acids produced the results shown in Table 9. Pure L-isomers were used where possible in the proportions found in casein. Otherwise, double quantities of the DL-isomer were given. Although a small proportion of flies with mature ovaries was produced by these diets, pure amino-acids were much less effective than whole protein. In order to show that amino-acids were not directly toxic, a diet containing both amino-acids and whale-meat juice was given. Such a diet gave ovarial development equal to that of the whale-meat juice control diet. Therefore, although a mixture of amino-acids may

be more difficult to metabolize efficiently, its presence in a diet does not affect the normal metabolic changes. (Considerable difficulty accompanied the feeding of a highly concentrated amino-acid mixture because of the insolubility of some constituents. Treatment with the Waring Blendor provided the most suitable diet, insoluble material being reduced to a suspension which could be ingested by the flies.) Use of a mixture containing DL-serine and L-isomers of the remaining amino-acids did not result in better development than that obtained with mixtures used for most of the above tests. The latter contained about 4 DL-isomers in addition to DL-serine. Therefore, unless DL-serine was strongly inhibitive (there was no evidence to favour this) the presence of small amounts of the unnatural isomer in the diet did not interfere with growth. When, however, a mixture containing mainly DL-isomers was tested and compensating double quantities were not given, there was no yolk formation in any fly. Since the effective concentrations of such amino-acids in this particular mixture had been reduced by half this suggests that, as with insects other than *Protophormia*, many D-isomers cannot be metabolized. (L-Isomers of all amino-acids have only since become commercially available.)

Table 9. *The result of replacing protein in the diet by equivalent mixtures of amino-acids*

Food given	Mean size		No. of flies	
	Fat body	Ovary	Total	Ovary, stage 5
Amino-acids 5 % (B) + Marmite 4 %	2·4	19,309	28	5
Amino-acids 6 % (C) + Marmite 4 %	2·3	12,264	36	12
Whale-meat mixture	2·3	24,460	21	14
Sucrose control	1·9	1,052	17	—
Amino-acids 5 % (G) + Marmite 4 %	2·3	12,963	36	12
Whale-meat mixture 5 %	2·1	33,055	34	31
Amino-acids 5 % (G) + whale-meat mixture 5 %	2·0	28,201	34	23
Amino-acids 2·5 % (G) + whale-meat mixture 2·5 %	1·8	30,865	38	34
Sucrose control	1·5	968	19	—
L-Amino-acids (E)	1·6	1,146	15	—
L-Amino-acids (E) + Marmite 4 %	2·3	8,018	30	4
DL-Amino-acids (F) + Marmite 4 %	1·9	1,833	25	—
Whale-meat mixture	2·0	39,125	6	6
Sucrose control	1·5	923	8	—

Bracketed letters refer to the amino-acid mixture used. See Appendix, Table 1.

(*d*) *Combination of protein and amino-acid mixtures.* It has already been shown that protein hydrolysates and pure amino-acid mixtures can only partially replace protein in the diet.

Although no record has been found where the existence of a non-specific substance present in proteins and not in amino-acid mixtures is proved, the following experiments were made to show whether the presence of small amounts of protein or other undialysable breakdown products of protein significantly improved ovarial growth in flies given pure amino-acids. In (*a*) a small proportion

of whole gelatin was added to a mixture of pure amino-acids, and in (*b*) further hydrolysis of the non-dialysable fraction of gelatin hydrolysate had been carried out. The results are shown in Table 10. In (*a*), each of the three foods gave a small percentage of flies with mature ovaries. When the amino-acid mixture was added to gelatin an increase, apparently additive, in the number of mature flies was produced. A two-factor analysis of variance of these results was made using angular transforms of the percentage of flies with mature ovaries. This showed that both gelatin and amino-acids were effective foods ($P < 0.05$), but that there was no interaction between them. This suggests that each food supplied similar factors in suboptimal amount. In (*b*) further hydrolysis of the non-dialysable fraction of gelatin hydrolysate did not reduce its food value. The slight decrease was non-significant. Both these experiments indicated that there was no factor, present in proteins but not in amino-acids, which was required by *Protophormia*.

Table 10. *To show that there is no substance present in proteins but not in their hydrolysates or equivalent amino-acid mixtures that is required for ovarial growth in the flies*

Food given	Mean size		No. of flies	
	Fat body	Ovary	Total	Ovary, stage 5
Gelatin hydrolysate 10%	2·1	14,244	34	11
Gelatin hydrolysate non-dialysable fraction 10%	2·0	9,725	35	6
Hydrolysed gelatin hydrolysate non-dialysable fraction 10%	1·7	12,026	38	9
Whale-meat mixture control 10%	2·0	19,725	8	5
Sucrose control	1·8	1,233	8	—
Gelatin 3% + Marmite 4% + tryptophane	1·9	6,311	20	2
Gelatin 3% + amino-acids 5% + Marmite 4%	2·3	18,087	30	14
Amino-acids 5% + Marmite 4%	2·1	8,416	29	6

Amino-acids = amino-acid mixture G.

(v) *Accessory factors*

It was apparent from earlier parts of this work that both adequate protein and accessory factors are essential for yolk formation. Such accessory factors were present in both meat and Marmite. These are relatively heat stable and are also dialysable. This was shown by the dialysis of whale-meat mixtures and also by the dialysis of Marmite solution (Table 11). In both cases of dialysis the dialysable fraction, together with a suitable protein, gave an excellent ovarial development.

However, considerable variation occurred among both the stock as a whole and among individual flies in the amounts and possibly also the nature of the substances required; for example dairy milk of the same grade produced different results in September and in February, although employed under the same conditions. Since experimental conditions were such that large amounts of food were

always ingested this might reflect seasonal differences in either the milk or the flies. Similarly, stocks of flies did not respond uniformly to standard diets of whale-meat, horse serum + Marmite, or gelatin hydrolysate + tryptophane + Marmite, although all these foods and particularly the latter had a reasonably constant composition. Moreover, individuals which had ingested similar quantities of the same food produced a widely varied response. The need for accessory factors may also be influenced by the bacteria present in the gut, but an investigation of this lay outside the scope of this work. Accessory factors are therefore essential for ovarial maturation, though the amounts required may be critical and appear to vary considerably with the condition of the stock and among individuals.

Table 11. *To show (a) that the accessory substances in Marmite occur in the dialysable fraction, and (b) that the artificial vitamin and salt mixture is not toxic*

Food given	Mean size		No. of flies	
	Fat body	Ovary	Total	Ovary, stage 5
Non-dialysable fraction	1·9	2,440	24	—
Dialysable fraction	2·1	15,584	32	11
Dialysable + non-dialysable fraction	2·2	14,005	27	9
Marmite 4%	2·2	16,675	30	9
Vitamins + salts	2·2	2,583	31	—
Non-dialysable fraction + vitamins + salts	2·0	2,419	30	—
Dialysable fraction + vitamins + salts	2·1	8,811	33	7
Dialysable + non-dialysable fractions + vitamins + salts	1·9	10,100	28	6
Whale-meat control	2·1	31,445	24	23
Sucrose control	1·4	1,089	22	—

Vitamin and salt mixture C were used throughout; for their composition see Appendix, Table 2.

Attempts were made to replace Marmite in the diet by a mixture of pure chemicals (Table 12). In compiling such diets the published analyses of Marmite and the known requirements of other insects for accessory factors were used as a guide.

No mixture satisfactorily replaced Marmite, although flies with mature ovaries were found in isolated cases and showed that at least some of the constituents were effective. Since the addition of 4% Marmite to such diets promoted ovarial development (Table 11) the physiologically large amounts of some substances included could not be considered toxic. Using such mixtures, however, the ovarial response was too small to permit the active and inactive components to be separated. Some essential factor remains to be identified.

The importance of vitamin B_{12} and penicillin was investigated separately (Table 12*c*) using a relatively ineffective synthetic diet. However, neither compound affected the distribution of flies with mature ovaries. As it may kill important gut bacteria, penicillin was also tested with diets of milk and whale-meat juice to see whether inhibition of yolk formation resulting from the addition of penicillin to the diet could be explained on this basis. The results of such tests are shown in Table 12*d*. Penicillin had no effect when given with whale-meat ($P > 0{\cdot}7$ by '*t*' test), neither

was the apparent reduction in ovarial growth produced by adding penicillin to milk significant ($P > 0{\cdot}3$ by 't' test). However, this test was not sensitive owing to the great range in ovary sizes for individual flies. It is therefore possible that penicillin had a small effect, which would be more noticeable in a milk diet where accessory factors are present in barely sufficient amount.

Table 12. *The effects of artificial vitamin and salt mixtures in diets containing adequate protein*

Food given	Mean size		No. of flies		
	Fat body	Ovaries	Total	Stage 5	Stage 4
(*a*) Egg albumen	2·7	3,347	36	4	—
Egg albumen + vitamins	2·5	3,534	35	4	—
Egg albumen + Marmite 4 %	2·1	21,637	37	32	—
Serum albumen	2·1	3,202	19	2	—
Serum albumen + vitamins	2·1	8,470	23	6	—
Serum albumen + Marmite 4 %	2·1	34,312	8	8	—
Milk	1·8	15,266	17	9	—
Milk + vitamins	2·0	5,848	25	4	—
Milk + Marmite 4 %	1·8	27,225	18	16	—
Whale-meat mixture	2·4	24,247	16	13	—
Sucrose control	1·5	882	20	—	—
(*b*) Gelatin hydrolysate amino-acids + vitamin mixture	2·2	2,661	66	2	6
Whale-meat mixture	2·1	7,230	8	2	1
Sucrose control	1·9	1,003	8	—	—
(*c*) Gelatin hydrolysate amino-acids + vitamins	2·3	4,680	16	—	3
+ penicillin	2·6	3,273	19	1	—
+ B_{12}	2·5	3,116	17	2	—
+ penicillin + B_{12}	2·6	1,757	18	—	2
Whale-meat mixture control	2·5	35,481	8	8	—
Sucrose control	2·0	788	12	—	—
(*d*) Whale-meat mixture + penicillin	1·8	14,214	18	8	—
Whale-meat mixture	1·8	16,249	18	10	—
Milk + penicillin	2·0	7,152	21	5	—
Milk	1·8	11,055	11	4	—
Sucrose control	1·3	702	20	—	—

(*a*) Vitamin and salt mixture C. (*b*) Vitamin and salt mixture X. (*c*) Vitamin and salt mixture X (×8 concentration). (*d*) Sodium penicillin, 25 %. For composition of vitamin and salt mixtures see Appendix, Table 2.

IV. DISCUSSION

In this account many conclusions already reached by former workers have been substantiated. Growth of the ovaries on a carbohydrate diet is strictly limited, and although adult fat body is formed normally it never reaches its maximum size. Further growth of the ovaries and yolk formation require considerable quantities of protein and traces of accessory substances. Protein-containing foods must be concentrated and soluble enough to allow ingestion of considerable quantities. 0·4 mg. protein is a suggested maximum requirement for each fly. Pure protein and sucrose alone never produced ovaries in advance of stage 3. In contrast Rasso & Fraenkel (1954) report that limited yolk formation occurred with dialysed sodium caseinate. This can only be reconciled as a species difference (*Phormia regina* was

used) or, as the former workers have suggested, the precautions against bacterial contamination of the food were not adequate.

Differentiation of the primary egg chambers and initial enlargement of the ovaries occur in sucrose-fed flies in the first 4 days of adult life, when the pupal fat body is disappearing. These processes have been correlated with the activities of the side lobes of the ring gland which persists until the fourth day of adult life (Thomsen, 1952). Since little further growth of either the ovaries or fat body occurs when a protein diet is withheld this early growth must depend on food reserves formed during larval stages and preserved in the pupal fat body. Pupal fat body, however, disappears more rapidly when a complete diet is given. This may be correlated with an increased metabolic rate since the corpus allatum, which has been shown by Thomsen (1949) to influence metabolic rate, also increases in size during this time. Moreover, the adult fat body reaches a maximum size during yolk formation and is smaller in the mature fly. The simultaneous decrease in size of fat body and enormous increase in ovarial size which occurs during yolk formation indicate that a transfer of food reserves from the fat body to ovary takes place. Since the gonadotrophic function of the corpus allatum hormone has been established it would be interesting to discover if its effect is to control this transfer of food material, as in the grasshopper *Melanoplus differentialis* (Pfeiffer, 1945).

The investigation of specific amino-acid requirements has been hindered by the difficulty in procuring a diet from which individual amino-acids could be eliminated, but it has been possible to show that tryptophane is essential. Pure protein could not be entirely replaced by equivalent amino-acid mixtures, ovarial growth being reduced by at least half. This reduction was not due to toxicity or to the presence of D-isomers. Growth was, however, greatly restricted when a mixture containing predominantly DL-isomers of amino-acids was used. This suggests that, as with other insects, D-isomers are not always utilizable. It was found that the relative amounts of amino-acids in the diet are very critical when unnatural mixtures are used. Unsuitable proportions may have been the reason for the inadequacy of the mixtures of pure amino-acids used here.

There was no evidence of the requirement of a specific factor present in proteins but not in hydrolysates of proteins or pure amino-acid mixtures. Addition of amino-acid mixtures to dilute protein diets gave an additive but not synergistic effect. The importance of amino-acid balance already noted was emphasized when the proportions of dialysable to non-dialysable fractions of gelatin hydrolysate were varied, and it was found that presence of a higher proportion of the inadequate dialysable fraction reduced ovarial growth although no reduction in the concentration of the non-dialysable fraction had been made. This is comparable to the 'relative lysine deficiency' found by Fraenkel (1948) in *Tribolium infestans* and the 'relative tryptophane deficiency' found by Maddy & Elvehjem in rats (1949). Resolution of this problem in the blowfly will not be possible until an accurate analysis of the amino-acid content of the two fractions has been made.

Accessory factors were less satisfactorily investigated owing to the failure to produce a chemically defined diet. Some essential factor or factors was/were still

missing from the vitamin and salt mixtures tested, although almost every substance known to be important for growth of other insects had been included. It would seem that a new factor is involved. Sodium penicillin had no marked effect on the efficacy of any diet and vitamin B_{12} did not improve growth on a synthetic diet. The complete vitamin and salt mixture did, however, contain some active constituents, but in the absence of essential substances their individual effects were too small to make their investigation practicable.

In contrast to Evans's (1936) results with *Lucilia*, ovarial development at 55% R.H. under optimum feeding conditions was as good as at 95% R.H., although flies were very much more active at the lower humidities. It was also surprising to find no correlation between the weight of a fly at emergence and its ovarial size at any time. However, ovarial size at emergence may be governed by genetic factors and not by the larval conditions which are very important in determining body weight.

There were considerable variations both in the behaviour of the stock at different times and in the individual flies which had been given the same amount of food. In some an apparent genetic abnormality resulted in the failure of the ovaries to grow beyond stage 1. In one winter stock a tendency to an accumulation of fat body with failure of ovarial growth on a complete diet was noted. This could be interpreted as an adult diapause. Such flies responded to much higher concentrations of Marmite or whale-meat juice than those normally needed, and apparently required unusually large amounts of the accessory factors for ovarial development.

V. SUMMARY

1. A method has been developed for obtaining 200–300 flies daily from a culture of *Protophormia terrae-novae* reared on whale-meat.

2. Normal growth changes in adults have been described, and five stages in ovarial development have been defined.

3. Experimental conditions affecting ovarial growth in adults have been investigated and a satisfactory routine method for examining the effect of adult nutrition on ovarial growth has been devised. Precautions were taken against bacterial contamination of food although non-sterile conditions were used.

4. Ovarial development was not related to relative humidity, if above 55% R.H., nor to the weight of the adults at emergence.

5. Evidence has been given for the conversion of food material from pupal to adult fat body and for the transfer of material from fat body to ovaries at yolk formation.

6. Healthy adult females can be maintained on a diet of sucrose and water. Addition to the diet of protein or amino-acids produces only slight enlargement of the ovaries. Yolk formation only occurs if the diet is further supplemented by accessory factors, or substances required only in small amount. Ovarial maturation requires considerable amounts of protein, though not more than 0·4 mg. per fly is needed and food solutions must be concentrated. The accessory factors have not

been identified but are known to be resistant to slow boiling and are dialysable. Certain mineral salts and B vitamins are thought to be involved.

7. Tryptophane is an essential part of the amino-acid mixture.

8. Protein hydrolysates or equivalent amino-acid mixtures can be substituted for the whole protein but are less effective. The proportions of amino-acids present are very critical, particularly when the amino-acids are in a free state. The amino-acid mixtures have no toxic effect and the presence of some D-isomers do not inhibit growth, although some D-isomers cannot be used by the flies for growth.

9. A chemically defined diet similar to that used for *Drosophila* did not promote ovarial growth in *Protophormia*. A mixture containing ten B vitamins, ribose nucleic acid and mineral salts and cholesterol contained some effective accessory substances but lacked some unidentified factor required for yolk formation. The missing factor was not vitamin B_{12}. Penicillin had no significant effect on ovarial growth whether used in minute or in antibiotic concentration.

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APPENDIX

Table 1. *Composition of amino-acid mixtures to show relative amounts used*

	Amino-acid mixture					
	Casein	B	C	E	F	G
Arginine	4·1	4·2	4·2	8·8	8·8*	4·2
Histidine	2·5	3·2	3·2	3·2	3·2	3·2
Lysine	6·9	7·5	7·0	2·4	2·4	7·0
Tyrosine	6·4	5·4	5·4	5·4	5·4*	5·4
Tryptophane	1·8	5·0	5·0	5·0	5·0	5·0
Phenylalanine	5·2	0·3	3·0	3·0	3·0	3·0
Cystine	0·36	0·4	1·0	1·0	1·0*	1·0
Methionine	3·5	3·5	3·5	3·5	3·5	3·5
Serine	7·5	6·8	8·5	6·5	6·5	6·5
Threonine	3·9	4·5	2·0	2·5	2·5	2·5
Leucine	12·1	9·0	8·5	8·5	8·5	8·5
iso-Leucine	6·5	7·0	7·0	7·0	7·0	7·0
Valine	7·0	7·7	7·7	7·7	7·7	7·7
Glutamic acid	22·8	11·0	12·0	12·0	12·0*	12·0
Aspartic acid	6·3	7·0	7·0	7·0	7·0	7·0
Glycine	0·5	2·1	2·1	4·1	4·1	2·1
Alanine	5·6	3·3	3·3	3·3	3·3	3·3
Proline	8·2	10·1	9·1	9·1	9·1	9·1
Hydroxy-proline	2·0	2·0	2·0	2·0	2·0*	2·0

All amino-acids used were the L-isomer except for methionine, serine, threonine and *iso*-leucine in mixtures B, C, E and F where the DL-isomer was used in double the amount shown and in mixture G where all amino-acids were of the DL-isomers except those marked * which were of the L-isomer.

APPENDIX (*cont.*)

Table 2. *Composition of Marmite and artificial vitamin and salt mixtures used*

Substance	Mixture C (mg. %)	Mixture X (mg. %)	Marmite 4 % (mg. %)
Vitamins			
Aneurin	0·9	0·12	0·12
Riboflavin	1·63	0·22	0·24
Nicotinic acid	0·425	1·1	2·4
Pantothenic acid	1·9	0·28	0·24
Folic acid	1·9	0·28	0·24
Pyridoxin	1·2	0·16	0·16
p-Amino benzoic acid	6·45	3·3	—
Biotin	0·04	0·02	0·004
Choline chloride	33·9	8·8	17·6
Inositol	17·3	4·3	7·2
Cholesterol	32·2	8·3	—
Ergosterol	32·2	41·8	—
Uracil	161·3	20·9	—
Adenine	80·6	20·9	—
Adenosine	80·6	23·3	—
Guanine	85·1	150·0	—
(Penicillin)	—	10 γ	—
(Vitamin B_{12})	—	—	—
Mineral salts			
Calcium phosphate	840	3000	—
Sodium chloride	210	750	—
Potassium chloride	140	500	—
Calcium carbonate	123·2	440	—
Magnesium carbonate	58·8	210	—
Ferrous sulphate	21·0	65	—
Manganese sulphate	3·5	12·5	—
Copper sulphate	1·4	5·0	—
Calcium iodide	0·7	2·5	—
Zinc oxide	0·7	2·5	—
Cobalt chloride	0·7	2·5	—

THE THERMAL SENSITIVITY OF THE LATERALIS ORGANS OF *XENOPUS*

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(*Received* 16 *July* 1956)

INTRODUCTION

During an investigation of the impulse discharge from the lateralis organs of *Xenopus laevis* (Daudin) a response to temperature change was found, and is here described. The lateralis organs of *Xenopus* are sensitive, in behavioural experiments, to slight mechanical disturbances in the surrounding water (Kramer, 1933; Dijkgraaf, 1947); the electrophysiological aspects of this sensitivity are known neither in *Xenopus* nor in any other amphibian, except that Dijkgraaf (1952), in a review of acoustico-lateralis organs, reported that Elbers had found in the lateralis nerves of *Xenopus* a spontaneous discharge increased by mechanical stimulation and having a post-stimulatory 'silent' period. But predictions can be made from the behaviour of the homologous organs of fishes. The characteristics of lateralis organs are their basic discharge, great sensitivity to stimuli which are effective in bending the sensory hairs (Hoagland, 1933*a*, 1934; Schriever, 1935; Sand, 1937; Suckling & Suckling, 1950; Katsuki, Yoshino & Chen, 1951), and, in elasmobranchs at any rate, inhibition of the basic discharge by stimuli which bend the hairs the other way (Sand, 1937).

The basic frequency varies directly with temperature (Hoagland, 1933*b*; Sand, 1938), but a thermoreceptor function of the lateralis organs of fishes (Hoagland, 1935) is unlikely (Dijkgraaf, 1940). A response to temperature change was found by Sand (1938) in the lateralis organs of *Raja*, but its extent was slight compared with the response to mechanical stimulation. The thermal response was similar to the much more sensitive response of the ampullae of Lorenzini, for there was a transient slowing of the discharge on heating and a corresponding increase of frequency on cooling.

The anatomy and innervation of the lateralis organs of *Xenopus* are described by Murray (1955).

MATERIAL AND METHODS

The experiments were performed on adult female *X. laevis* imported from South Africa for use in pregnancy diagnosis tests and maintained in tanks at 20° C. in the laboratory for up to 2 years. They were killed by pithing through the nares (Murray & Russell, 1952).

An isolated preparation was employed, consisting of a small piece of skin

approximately 12 mm. in diameter from the scapular region together with up to 20 mm. of the lateralis branch of the vagus nerve. Cuts were made in the skin in the mid-dorsal line and transversely behind the eye and over the middle of the scapula; the flap of skin so formed was pinned out and the lateralis nerve freed inwards to its origin from the skull where it was cut, and the flap of skin was removed from the body. The nerve was cleared of other tissues and all its branches except that running to a single group of lateralis organs were cut. The average number of active units in such a preparation was two.

An *in situ* preparation involving the anterior lateralis nerve to the circumorbital row of organs was also used, but the length of nerve available was short and only multifibre records were obtained, which confirmed the results obtained with the isolated preparations.

Experiments were also made with similar-sized, isolated pieces of trunk skin together with the dorsal or lateral segmental cutaneous nerves which ran to them.

The physiological saline used was to the formula of Landgrebe & Waring (1944).

For recording, a conventional, condenser-coupled, balanced amplifier was used, with presentation on a cathode-ray oscillograph and loudspeaker; for some of the experiments a counting-rate meter was available (Groen, Lowenstein & Vendrik, 1952), from which the impulse frequency could be read off at any time. This otherwise most useful instrument had the one disadvantage that it did not respond immediately to sudden changes of impulse frequency, and therefore the maximal frequency of a rapidly adapting outburst was underestimated. Permanent records were made photographically, at first by a single-shot camera. With this more than one sweep of the time-base could be recorded by triggering a slow 'Y' shift with the same circuit that opened the camera shutter (Fig. 1).

Thermal stimuli were presented by passing water of known temperature beneath a cover-glass on which the preparation, enclosed in a moist chamber, was placed. The time-course of typical changes of temperature was measured by placing thermocouple junctions between the skin and the cover-glass and on the inner surface of the skin. The rate of change of temperature at the outside of the skin started with a maximal value of 0·3° C./sec. for each 1° C. of final change of temperature and thereafter decayed with a time-constant of less than 5 sec. The gradient across the skin reached its maximum after 5 sec. and thereafter decayed with a time-constant of approximately 15 sec. During an experiment a suitable sequence of temperature changes was carried out, normally with at least 2 min. between each change to allow the discharge frequency to become steady at its new level.

Twenty-five animals were used for these experiments, many of them yielding two isolated preparations.

RESULTS

A basic discharge occurs in the majority of units. Relatively few are normally silent and can only be identified on stimulation. The discharge is irregular, especially at low frequencies (Fig. 1); the average frequency of single units at 18–20° C. is 20/sec.

The basic discharge (measured immediately before a temperature change when it is steady) varies directly with the temperature, having a coefficient (Q_{10}) for 10–20° C. between 2 and 3; the basic discharge is not consistent enough for an accurate calculation to be made (Fig. 2).

The response to temperature change is similar to that described by Sand (1938) in *Raja*: a fall of temperature is followed by an increase in frequency of discharge, and a rise in temperature by a corresponding slowing-down or even cessation, the discharge in both instances reverting to the new steady level with a time-constant of

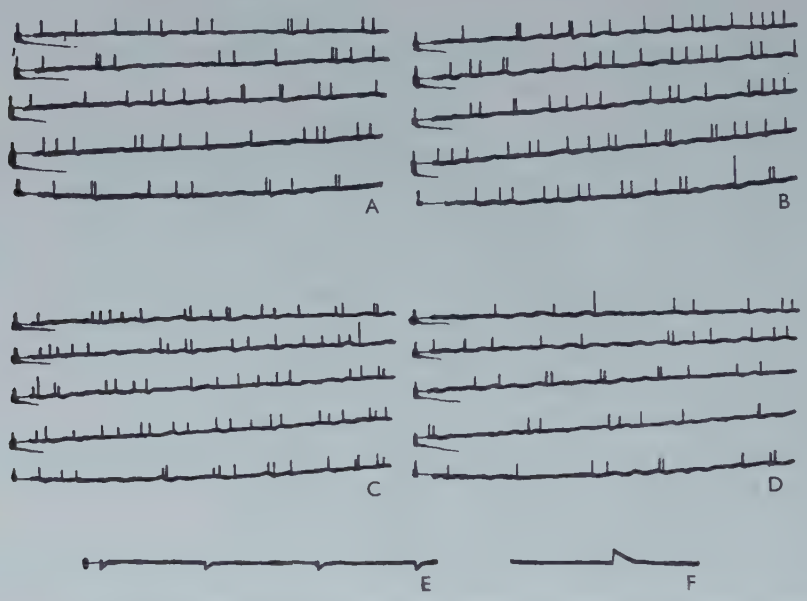

Fig. 1. Action potentials recorded from an isolated, two-fibre lateralis preparation, showing the irregularity of the basic discharge and the response to temperature change. A, at 20° C.; B, 5 sec. after change to 15° C.; C, at 16° C.; D, 5 sec. after change to 23° C.; E, time trace (0·1 sec.). F, 50μV. calibration. The records read from left to right and from bottom to top. (Traced from photographs.)

about 20 sec. (Figs. 1, 3). This reversed response is found in all preparations. A control experiment in which the water was changed in the usual way but without change of temperature was without effect, and this confirmed that it was the changes of temperature which were responsible for the response.

Because of the irregularity of the basic discharge the threshold rate of change of temperature cannot be determined accurately, but a change of 1–2° C., which represents a rate of change of 0·3–0·6° C./sec., normally causes a detectable change in the record. The full stimulus-response relationships of two representative preparations are shown in Fig. 4A, B. The points represent the maximal (or minimal) frequency immediately after the change of temperature expressed as a proportion of the steady value immediately before the change; this method of expression is the only one which can take account of the wide differences in the initial basic rate due to

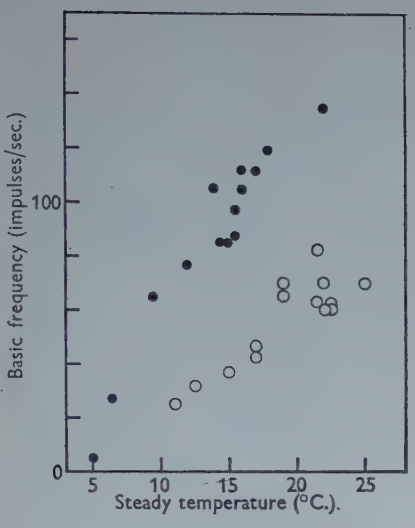

Fig. 2

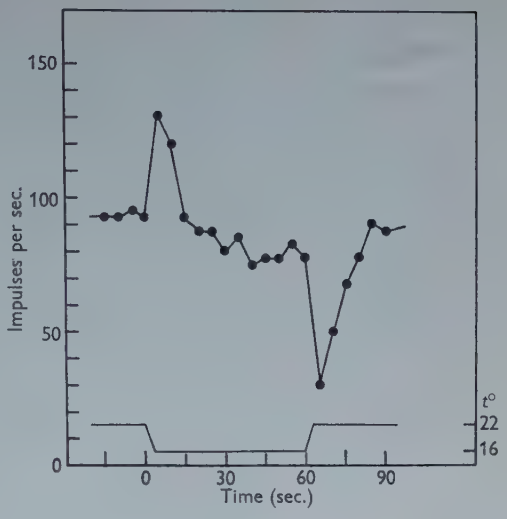

Fig. 3

Fig. 2. Impulse frequencies in two isolated multifibre lateralis preparations as a function of steady temperature.

Fig. 3. Impulse frequencies of an isolated multifibre lateralis preparation during a fall and rise of 6° C. Upper trace: impulse frequency recorded by meter. Lower trace: temperature.

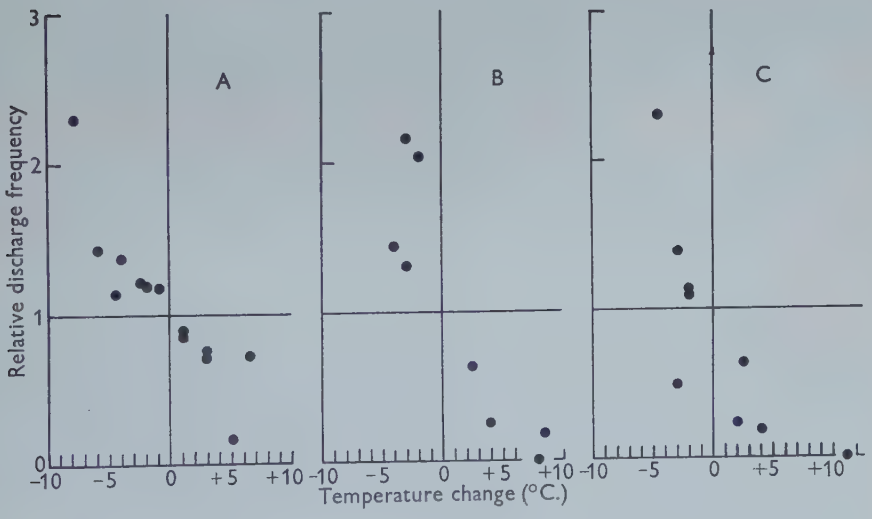

Fig. 4. The responses of three isolated multifibre lateralis preparations to temperature change. A, B, temperature changes applied from outside skin; C, temperature changes applied from inside skin. The maximal or minimal frequency immediately after the change, expressed relative to the preceding basic frequency, is plotted against the magnitude of the change.

differences of initial temperature and age of the preparation; it also allows preparations with different numbers of active units to be compared directly with one another. On average the responses to warming and cooling are symmetrical. The responses of many preparations are plotted together in Fig. 5.

A further experiment was performed to determine whether it was the spatial gradient across the skin or the temporal course of the change of temperature which was effective in causing the response. A typical series of changes was applied to a preparation mounted upside-down on the cover-glass, so that the inside face of the skin was towards the water and the nerve ran up to the electrodes from under the edge of the skin. The results of this experiment are shown in Fig. 4C, and it is clear that the reversal of the spatial gradient does not alter the sign of the response.

When tested in a similar way to the majority of the lateralis preparations, segmental cutaneous nerve preparations require changes of temperature of between 5 and 10° C. fall and over 10° C. rise before responses are detected in the nerve.

In response to mechanical stimulation both *in situ* and isolated preparations were most insensitive. Slight disturbances which would have produced a marked response in *Raja* (Sand, 1937) and comparable with those described by Kramer (1933) were quite ineffective. In order to produce an increase in the discharge rate recognizable over the loudspeaker the stimulus had to be such that the skin could be seen to be distorted; pipette-jets of water or touches on the skin a few millimetres away from the organ were examples of such relatively massive stimuli. There was no difference between experiments in which the skin was pinned with its inner face to the wall of a container of saline and those in which the skin formed a diaphragm separating water from saline. The use of a mild anaesthetic (1% urethane) which reduced the necessity of handling the toad, followed by the minimum of dissection required to expose and record from the lateralis branch of the vagus did not result in greater sensitivity; this concentration of anaesthetic did not abolish the basic discharge.

When tested in a similar way segmental cutaneous nerve preparations give typical anuran rapidly adapting responses, and their sensitivity is of the same order as that of the lateralis preparations, an actual touch or distortion of the skin being required.

DISCUSSION

The existence of the basic discharge in the lateralis nerves of *Xenopus* was expected from the evidence of other homologous organs. It can also explain Kramer's (1933) finding that for a short period after operation toads with the lateralis organs of one side of the head cauterized would respond to stimulation from ahead by swimming towards the intact side; later they would orientate correctly and swim straight towards the source. This suggests a process of central compensation for the lost peripheral activity comparable to that found in the labyrinthine posture system (Bechterew, 1883).

The problem of whether the thermal sensitivity has any importance in the life of the animal cannot be answered by electrophysiological investigation. The most that can be said is that the temperature effect is likely to be significant compared with

other effects produced in the nerves. This can be illustrated by a comparison of the results described here for *Xenopus* and those of Sand (1938) for the ampullae of Lorenzini and the lateralis organs of *Raja* (Fig. 5). It can be seen that in general the more sensitive *Xenopus* preparations respond with either a doubling or a complete inhibition of the basic discharge to a change of temperature of 5° C. The *Xenopus* preparation therefore is intermediate in sensitivity between the two

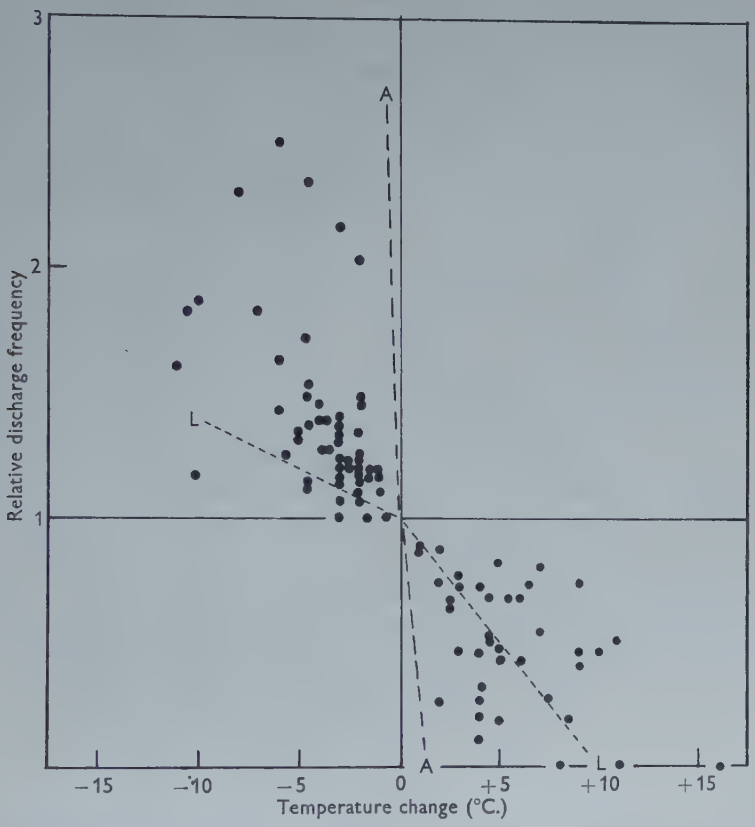

Fig. 5. The responses of all the lateralis preparations to temperature change, expressed as in Fig. 4. The responses of the most sensitive ampullae of Lorenzini (— — —, A) and lateralis organs (– – –, L) of *Raja* (Sand, 1938) are added for comparison.

Raja ones. In his discussion Sand concluded that the response of the ampullae was sufficiently great to warrant their description as organs specific for temperature change; but the similar behaviour of the lateralis organs was not considered significant compared with their specific function as mechano-receptors. The moderate sensitivity of the lateralis organs in *Xenopus* makes their function as thermoreceptors unlikely, even though they were found to be more sensitive than the segmental nerves.

Whether they are thermoreceptors or not, an interesting problem arises. *X. laevis* lives in a habitat (ponds, streams, ditches, etc.) in which marked tem-

perature fluctuations are likely. These fluctuations would cause changes in the discharge frequency from all the lateralis organs, and these changes would have to be distinguished by the central nervous system from the effects of mechanical stimulation. Presumably this distinction would be made possible by the different spatio-temporal patterns of the afferent nerve impulses.

The fact that the response to change of temperature is the same whichever way the spatial gradient lies means that theories of temperature reception based on the importance of a spatial gradient (Tyrrell, Taylor & Williams, 1954) cannot hold for *Xenopus*. For cooling the outside of the skin from 20 to 15° C. must establish a gradient similar in direction to that produced by warming the inside from 15 to 20° C., yet the response in the nerves will be an increase in frequency after the former and a decrease after the latter. If therefore nerve endings sensitive to temperature change are known in which the response does not depend on the spatial gradient, there is less reason to postulate the latter type of mechanism in mammals (Lele, Weddell & Williams, 1954).

The lack of sensitivity to mechanical stimuli comparable with that found by Kramer and Dijkgraaf in their behavioural experiments must presumably be explained by damage to the sense organs. The most probable site for this damage is the cupula, for the nerve endings themselves—the region of origin of the basic discharge—retain their spontaneity and their ability to respond to temperature changes; the recorded responses of the preparation to mechanical stimulation would then be due to distortion of the nerve endings themselves without the intervention of the cupula-sensory cell system. It must be assumed that even the minimal amount of handling required to obtain electrical records is sufficient to reduce the great sensitivity of the intact system. The lateralis organs are of course more likely to suffer damage in handling in *Xenopus* where they lie open at the surface of the skin than in those fishes in which the organs are enclosed in a canal. Electrical responses from free neuromast organs in fishes have not so far been recorded.

SUMMARY

1. A convenient isolated preparation of the lateralis organs and nerve of *Xenopus laevis* is described. An average of two active units can be obtained by cutting between the groups of organs in the skin.

2. There is a basic discharge, which is irregular and which varies directly with steady temperatures.

3. An opposite response is obtained to temperature changes. On cooling there is an increase in frequency adapting to the new steady level with a time-constant of 20 sec., and on warming there is a corresponding decrease in frequency.

4. The threshold for temperature change is 1–2° C., representing a rate of change of temperature of 0·3–0·6° C./sec. at the surface of the skin.

5. In the more sensitive preparations the basic frequency is doubled or completely inhibited by a change of 5° C.

6. The threshold change of temperature for similar preparations of skin and segmental cutaneous nerve is 5–10° C.

7. The discharge frequency can be increased by mechanical stimulation, but only at intensities high enough to act at the nerve endings directly and not by way of the sensory cells. The sensitivity is comparable to that of the segmental cutaneous nerves.

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